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Effects of Shiga toxin 2 on isolated porcine granulocytes

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
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Ames, Iowa

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ABSTRACT

Shiga toxin (Stx) binding to polymorphonuclear neutrophils (PMN) is hypothesized to play a role in the pathogenesis of Shiga toxin-producing *Escherichia coli* (STEC) disease in humans. Pigs are an excellent model for studying the role of PMN in STEC disease because, like humans, they are susceptible to Stx-mediated disease and Stx binds to porcine PMN *in vitro*. The objectives of this study were to develop a flow cytometric assay to monitor Stx2 binding under conditions required for functional studies (37°C in cell culture medium) and to evaluate the functional effects of Stx2 on porcine granulocytes. Binding and internalization of Stx2, or the non-toxic binding subunit of Stx2 (Stx2B), by isolated granulocytes, and the effects on apoptosis and oxidative burst were monitored by flow cytometry. Granulocyte preparations contained PMN and eosinophils (EOS) which bound and internalized Stx2 and Stx2B. Similar percentages of Stx2+ granulocytes were detected when Stx2 incubation was performed at 4°C and 37°C. Incubation with Stx2, but not with Stx2B, increased the mean percentage of apoptotic cells and reduced the mean percentage of cells capable of an oxidative burst. Pre-incubation of Stx2 with monoclonal antibodies against the Stx2B prevented Stx2 binding and effects on apoptosis and oxidative burst. The demonstration that Stx interacts with both porcine PMN and EOS *in vitro* extends the evidence that granulocytes, may contribute to STEC pathogenesis. The availability of a porcine Stx-mediated disease model will facilitate further investigations of the role of granulocytes in STEC disease.

CHAPTER 1: INTRODUCTION

General Introduction

Shiga toxin-producing *E. coli* (STEC) cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans worldwide. HUS is a life-threatening systemic complication of STEC infection and the leading cause of acute renal failure in children (98). Shiga toxins 1 and 2 (Stx 1 and 2) are important virulence factors thought to be the major mediators of STEC-induced HUS (6). These potent cytotoxins bind to globotriaosylceramide (Gb_3/CD77) receptors on mammalian cells, but the precise sequence of events leading from intestinal colonization with STEC to the development of systemic complications is not known. Numerous studies indicate the involvement of polymorphonuclear neutrophils (PMN) (9, 10, 29, 32, 48, 59, 60, 72, 77, 118, 119, 120). However, the specific role of these inflammatory cells in this process is not known. An increased understanding of the role of PMN in the pathogenesis of Stx-mediated disease will facilitate development of intervention strategies.

PMN may contribute to the development of systemic complications during STEC infection by allowing Stx to reach circulation and carrying the toxin to target organs including the kidney. Transmigration of human PMN into the intestinal lumen in response to Stx-induced inflammatory signals (124, 125) facilitates the movement of Stx into the bloodstream (60) and Stx bound to circulating PMN can be detected in blood from human patients with HUS (9, 117, 120). Human PMN do not express the Stx receptor Gb_3/CD77, but appear to bind Stx via a lower affinity receptor (48, 119). The ability of PMN to transfer

bound Stx to sensitive cells expressing Gb_3 (119) as well as to other PMN (10) *in vitro* provides evidence that PMN may play a role in transporting Stx to the kidneys and CNS. Stx2 binds to isolated human PMN and delays the onset of apoptosis in these cells (10, 77). A delay in PMN apoptosis may prolong the functional lifespan of these cells and the amount of time they are in circulation. Stx also triggers the PMN oxidative burst that may result in tissue damage (72).

Pigs are useful models for studying the role of Stx in disease development. Weaned pigs are susceptible to a naturally-occurring and experimentally-induced disease known as edema disease caused by STEC strains that produce Stx2e (17, 61). Neonatal pigs develop Stx-mediated disease following inoculation with STEC strains that produce Stx1, Stx2 or Stx2e (19, 20, 38, 50, 95). Systemic effects of Stx in pigs are similar to those seen in humans and include CNS (24, 38, 127) and kidney (50, 95) lesions. Currently, the mechanism by which Stx reaches circulation and the target organs in these animals is unknown. Stx binds to porcine peripheral PMN *in vitro* (136). However, the functional effects of this binding have not been identified.

The overall objectives of this study were to develop a flow cytometric assay for evaluating Stx2 binding to isolated porcine granulocytes under conditions suitable for granulocyte functional studies and to investigate the effects of Stx2 on porcine granulocyte functions, namely apoptosis and oxidative burst.

Objectives and Specific Aims

Statement of the research problem

The functional effects of Stx binding to porcine granulocytes *in vitro* have not been investigated.

Objective 1

Develop a flow cytometric assay for evaluating Stx2 binding under conditions required for functional studies.

Specific aims:

1) Establish a flow cytometric Stx binding assay for use at 4°C.

The previously described rapid flow cytometric assay for detecting Stx binding to isolated porcine granulocytes (135) was designed as a rapid procedure in which primary and secondary detection antibodies are combined in the same incubation step. Although the rapid staining procedure reduces handling of the cells and the time needed to complete the assay, there may be a possibility of cross-reactions or reduced staining when the detection reagents are combined compared to a sequential assay procedure in which separate incubation and wash steps are used for each reagent.

Hypothesis: The rapid and sequential Stx2 binding detection assays produce similar results.

2) Optimize the Stx binding assay for use under conditions required for functional studies using incubation of granulocytes and Stx2 at 37°C in cell culture media.

Functional assays must be carried out at 37ºC in cell culture medium. An important step before examining the functional effects of Stx2 on porcine granulocytes is to ensure that Stx2 binds to granulocytes under the required conditions. The flow cytometric Stx binding

assay will be optimized for use at 37ºC by determining the effects of incubation time, temperature, and medium on Stx2 binding.

Hypothesis: Stx2 binds to isolated porcine granulocytes under conditions needed for functional studies.

3) Use flow cytometry to evaluate internalization of Stx2 and rStxB2 by porcine granulocytes.

The Stx2 holotoxin targets the 28S rRNA and is by cells before it can exert any toxic effects. Although the StxB2 binding subunit has no toxic activity, it can affect cells through signaling pathways beginning with receptor binding at the cell surface. Internalization studies may yield information on how any functional effects of Stx2 or rStxB2 are mediated.

Hypothesis: Stx2 and rStxB2 are internalized by isolated porcine granulocytes.

Objective 2

Determine the effects of Stx2 on porcine granulocyte function.

Specific Aims:

1) Determine the effect of Stx2 on apoptosis of isolated porcine granulocytes.

Stx delays apoptosis in human granulocytes possibly extending their functional life span and increasing the amount of time the cells are in circulation. Delayed apoptosis may allow the transport of Stx to target organs and result in an increase in granulocyte-mediated tissue damage. Since pigs develop kidney and CNS lesions comparable to those observed in humans, Stx may have similar effects on apoptosis in porcine and human granulocytes.

Hypothesis: Stx2 delays apoptosis of isolated porcine granulocytes.

2) Determine the effect of Stx2 on porcine granulocyte oxidative burst.

Stx increases the oxidative burst of human granulocytes and inhibits their ability to respond to subsequent stimulation with phorbol 12-myristate 13-acetate (PMA). Stx2 may have a similar effect on the oxidative burst of porcine granulocytes.

Hypothesis: Stx2 decreases the percentage of PMA-stimulated porcine granulocytes capable of an oxidative burst.

CHAPTER 2: LITERATURE REVIEW

Introduction

Some Shiga toxin producing *E. coli* (STEC), including serotype O157:H7, cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. HUS is a potentially fatal condition characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. Of the estimated 70,000 cases of STEC infection in the United States each year (83), up to 10% develop HUS. HUS most often occurs in young children and is the leading cause of acute renal failure in children in the United States (98). Healthy cattle are considered the primary reservoir for STEC (8) and undercooked meat, dairy, and vegetables contaminated with bovine feces are common sources of infection for humans (68).

Shiga Toxins

All STEC strains characteristically produce one or more potent cytotoxins called Shiga toxins (Stx) that are important virulence factors hypothesized to be responsible for the development of HUS (6). The two major types of Stx are Stx1 and Stx2 although several variants exist including Stx2c, Stx2d, Stx2e, Stx2f (63, 79, 84, 107). Stx1 and Stx2 are closely related to Shiga toxin produced by *Shigella dysenteriae* (89) and were initially named Verotoxins I and II or Shiga-like toxins I and II (12). Stx1 is 98% homologous to Shiga toxin produced by *S. dysenteriae* and differs by only one amino acid (114). In contrast, Stx2 shares only 56% sequence homology with Stx1 (66). Stx1 and Stx2 are antigenically distinct (112). Stx1 is neutralized by antiserum against Shiga toxin from *S. dysenteriae*, but Stx2 is not

neutralized by antiserum against Stx or Stx1 (112). The genes for Stx1 and Stx2 are encoded by bacteriophages (90) while the closely related Stx from *S. dysenteriae* is chromosomally encoded.

Shiga toxins are AB toxins consisting of a 32kDa A (active) subunit non-covalently associated with five 7.7kDa B (binding) subunits (22). The A subunit is composed of two fragments, A_1 and A_2, joined by a disulphide bond (114) and proteolytic cleavage of the A subunit activates Stx (74). The A_1 fragment is a 28S rRNA N-glycosidase necessary for the enzymatic activity of Stx (40) while the A_2 fragment stabilizes the interaction between the A_1 and B subunits (3). The B subunits bind to specific receptors and studies with constructed hybrid toxins show that the receptor specificity of Stx is determined by the type of B subunit present (134). The A subunit of Stx2 but not Stx1 is accessible in the holotoxin and may also have a role in receptor specificity and binding (39).

Stx1 and Stx2 have a similar mechanism of action, but there are some differences between the two. Infection with STEC strains expressing Stx2 is more often associated with severe disease (6) and Stx2 is more likely to lead to systemic complications in pigs (24). Stx1 and Stx2 target different tissues and Stx2 has a lower LD_{50} in mice (101, 121). Stx1 and Stx2 bind different sites on the Gb_3 receptor (64). There are also differences in the intracellular transport of the toxins in some cell types (116).

The membrane glycolipids called globotriaosylceramide (Gb_3) and globotetraosylceramide (Gb_4) serve as the primary receptors for Stx on mammalian cells. Stx1 and Stx2 preferentially bind to Gb_3 (75, 132) while Stx2e, a variant of Stx2 that causes edema disease in pigs, preferentially binds to Gb_4 (21). The fatty acid content (94) and length of the fatty acid chain (71) of the glycolipid receptors affect their specificity and affinity.

Both the presence of Gb_3 on the cell membrane and the density of Gb_3 in the lipid rafts are important factors in Stx binding (51). The differential organization of Gb_3 in the lipid rafts may contribute to the age-associated susceptibility to HUS (70). Stx1 and Stx2 differentially bind to some Gb_3 isoforms and do not bind to all Gb_3 (14, 71, 76).

There is evidence that Stx can bind to certain types of cells in a Gb_3-independent manner. Stx appears to bind to a receptor with lower affinity than Gb_3 (119) on human polymorphonuclear leukocytes (PMN) and to Gb_3 as well as a second, unknown glycolipid receptor (15) on the surface of platelets. Stx modulates signaling pathways in a Gb_3-negative human intestinal epithelial cell line (46, 108).

Stx binds to glycolipid receptors via the B subunits and is subsequently endocytosed by many cell types (104). Stx binds uniformly over the cell surface at low temperatures but collects in clathrin-coated pits and is endocytosed when the temperature is increased (104). Although the preferential mechanism for Stx uptake involves clathrin-coated vesicles (106), a clathrin-independent mechanism has also been described for some cell types (88).

Once the toxin is internalized it follows one of several paths through the cell. Cells that are not sensitive to Stx route the toxin to lysosomes for degradation (106). In cell types that are sensitive to Stx, the toxin is routed through the cells in a retrograde manner. Stx enters endosomal compartments before being routed to the trans-Golgi network, Golgi stacks, and the endoplasmic reticulum. The Stx B-subunit is routed through many cell types to the endoplasmic reticulum in the same manner as the holotoxin (67, 105). Retrograde transport of proteins usually requires a KDEL (Lys-Asp-Glu-Leu) sequence but the Stx B subunit does not contain one (65). Several experiments indicate that retrograde transport is

not controlled by Stx but by the composition and length of the fatty acid chain of the glycolipid receptors (103).

Cleavage of the Stx A subunit into A1 and A2 subunits increases the activity of Stx (44, 74). Furin, a serine protease localized in the trans-Golgi network is the enzyme primarily responsible for this cleavage, but calpain or other enzymes in the cytosol may also be capable of cleaving the A subunit (43). Stx is transported to the cytosol by chaperone proteins in the endoplasmic reticulum (138) where it inhibits protein synthesis by affecting the 28S ribosomal RNA. Stx cleaves a N-glycosidic bond and releases an adenine base at the site of aminoacyl-tRNA docking, thereby inhibiting peptide chain elongation. This inhibition of protein synthesis quickly kills the cell (27).

Hemolytic Uremic Syndrome

In 5 to 10% of individuals infected with STEC, the disease will progress to hemolytic uremic syndrome (HUS), a condition characterized by hemolytic anemia, thrombocytopenia, and renal damage. HUS most often develops in pediatric and elderly patients and is the leading cause of acute renal failure in children in the U.S. (98, 100). Approximately 20% of HUS patients develop central nervous system (CNS) complications, including encephalopathy and stroke (91). The first symptoms of HUS usually appear 4-13 days after the onset of gastrointestinal symptoms although not all HUS cases are preceded by diarrhea.

Shiga Toxin and Hemolytic Uremic Syndrome

Stx plays a critical role in the pathogenesis of HUS. Histopathological analysis has revealed that Stx is cytotoxic to vascular and epithelial cells in the kidneys and to vascular cells in the central nervous system (CNS). Studies of Stx cytotoxicity *in vitro* reveal that Stx can damage renal vascular endothelial (93), mesangial (110), tubular epithelial cells (18), and cerebral microvascular endothelial cells (28). Bacterial lipopolysaccharide (LPS) acts synergistically with Stx to increase cytotoxicity (78). The variable expression of Gb_3 by different cell types may be responsible for the localization of damage to those areas expressing high numbers of the glycolipid receptors including the kidney and CNS (92, 122).

Stx also contributes to the development of HUS through the Stx-mediated release of cytokines and chemokines that contribute to the inflammatory response (54). Stx upregulates the expression of tumor necrosis factor alpha (TNF-alpha) and interleukin-1 beta (IL-1 beta) by macrophages and monocytes (52, 53). TNF-alpha (25, 92, 111, 131) and IL-1 (128) increase Gb_3 expression in endothelial cells and increase their sensitivity to the effects of Stx. Stx induces the release of TNF-alpha, IL-1, and other cytokines by renal tubule cells (57), glomerular epithelial cells (58), and cerebral microvascular endothelial cells (26), thus contributing to localized inflammation in the kidney and brain. Stx upregulates and stabilizes C-X-C chemokine mRNA, including IL-8, leading to an increase in production by intestinal epithelial cells *in vitro* (124, 125). This may increase the host inflammatory response and the influx of inflammatory cells into the intestine.

Thrombocytopenia and hemolytic anemia are characteristics of HUS. Stx-mediated inhibition of protein synthesis and death of sensitive vascular endothelial cells leads to detachment of the cells from the basement membrane. Underlying prothrombotic proteins

such as collagen are exposed and can initiate platelet aggregation and fibrin deposition (129). Stx binding to platelets *in vitro* leads to aggregation and binding of platelets to endothelial cell membranes (69). The resulting formation of microthrombi in the vasculature inhibit blood flow, particularly in the kidney, leading to ischemic damage and renal failure (13). Hemolytic anemia occurs when red blood cells are damaged in circulation, possibly by passage through capillaries and small vessels that contain microthrombi formed as a result of vascular damage (96, 98).

Shiga Toxins and PMN

PMN appear to be involved in the pathogenesis of STEC-mediated disease. HUS patients have elevated PMN counts that are indicative of a poor prognosis (11, 102, 133). An increase in circulating PMN is a risk factor for developing HUS (4) and correlates with the severity of renal impairment in HUS patients (30, 31, 32). High numbers of PMN are also found in the glomeruli of children with HUS (62). IL-8, an activator of neutrophils, appears to be partly responsible as it is elevated in HUS patients and correlates with an increase in the percentage of peripheral PMN (35). Increased levels of elastase and evidence of degranulated PMN in the peripheral blood of patients with HUS indicate that the PMN are activated (30, 32). PMN isolated from HUS patients readily adhere to and damage cultured endothelial cells (37, 86). Damage from activated, circulating PMN could increase endothelial cell injury and contribute to the development of the prothrombotic state in HUS.

Studies with animal models of HUS provide further evidence that PMN have a role in the development of Stx-mediated disease. Mice intravenously injected with Stx2 develop an increased percentage of circulating neutrophils with enhanced cytotoxicity and adhesive

properties (2, 34). The increase in neutrophils also positively correlates with the degree of renal damage in mice (34). Similar experiments in mice depleted of granulocytes showed reduced Stx2-induced renal damage and lethality (33). Dutch belted rabbits administered intravenous Stx2 develop high levels of circulating PMN and PMN infiltration of the intestine and kidney (42).

PMN may passively enable Stx transport from the intestinal tract to circulation. During STEC infections in humans, Stx upregulates the production of IL-8 and other chemotactic factors by intestinal epithelial cells, thus enhancing PMN infiltration (124, 125). PMN transmigration across polarized intestinal epithelial cells *in vitro* increases the movement of Stx across the cells in the opposite direction (60).

Stx bind to PMN *in vitro* and Stx bound to circulating PMN can be detected in the blood of children with HUS (9, 10, 117, 118). Although human PMN bind Stx, they do not express Gb_3 but are thought to express a different receptor with lower affinity (48, 119). PMN may be capable of transporting Stx to target organs. Human PMN pre-loaded with Stx transfer the toxin to human glomerular microvascular endothelial cells (GMVEC) resulting in inhibition of protein synthesis and cell death (119). PMN with bound Stx are also capable of transferring the toxin to Stx-negative PMN (10). Stx binds to other blood components including red blood cells (5), platelets (15), and monocytes (45, 97, 130) although these cells express Gb_3/CD77 and there is no evidence they are involved in Stx transport.

Several lines of evidence indicate that Stx has direct effects on PMN function *in vitro* although studies have produced some conflicting data. Incubation of human PMN with Stx delayed spontaneous apoptosis, extending the functional lifespan of the cells (10, 77) while other studies reported no effect of Stx on PMN apoptosis (36, 72). Stx induced an oxidative

burst in human PMN but Stx treated PMN were less responsive when phorbol 12-myristate 13-acetate (PMA) stimulation was used to elicit an oxidative burst (2, 72). Stx treatment also reduced the phagocytic ability of the PMN (72). Unlike human PMN, murine PMN express the Gb_3 and Stx increases the percentage of apoptotic murine PMN (47, 48). Stx increases the release of reactive oxygen intermediates (ROI) and impairs phagocytosis in murine PMN as they do in human PMN (47).

Shiga Toxin and Pigs

Pigs are susceptible to both natural infections with STEC strains that cause edema disease and to experimental infections with STEC strains that cause disease in humans. Edema disease, primarily a disease of weaned pigs, is caused by host-adapted STEC strains and is characterized by vascular necrosis, edema, and neurological complications (79). Stx2e, the Stx variant involved in edema disease, preferentially binds to Gb_4 instead of Gb_3 (21) and can be detected on circulating red blood cells in infected pigs (82). Edema disease has been used as a model to study the relationship between levels of Stx in the intestine and blood and the development of systemic complications (17). Pigs are a good model for studying the contribution of Stx to the development of disease because several features of Stx-mediated disease in pigs are similar to those in humans. Neonatal piglets inoculated intragastrically with STEC develop systemic complications (19, 20) and kidney lesions similar to those in humans with HUS (95). Gnotobiotic neonatal pigs develop brain lesions, vascular necrosis (38, 127), and thrombotic microangiopathy in the kidney (50). STEC producing Stx2 are more likely to lead to systemic disease in gnotobiotic pigs than strains that produce Stx1 or a combination of Stx1 and Stx2 (24). These results are similar to epidemiological data from

humans indicating that infection with STEC producing Stx2 is more likely to lead to severe disease and HUS. Pigs inoculated intravenously with Stx develop systemic disease including neurological signs and kidney lesions (41). Treatment of STEC-infected gnotobiotic piglets with antibody to Stx prevents the development of systemic disease (23, 87, 109). Stx1 and Stx2 bind to monocytes, alveolar macrophages, and to PMN *in vitro* (136).

Conclusions

PMN appear to be involved in the pathogenesis of Stx-mediated disease in humans, but their role is not well understood. Pigs are an excellent model for studying the role of PMN in STEC-mediated systemic disease. STEC-infected pigs develop STEC-mediated systemic disease characterized by kidney lesions and CNS complications similar to those seen in humans with HUS. Stx binds to PMN from pigs. The porcine model of Stx-mediated disease will facilitate further investigations of the interaction between Stx and PMN and identification of direct effects of Stx on PMN function and the role of PMN in the translocation of Stx out of the intestine, the transport of Stx to target organs, and the development of localized inflammation in target tissues.

CHAPTER 3: MATERIALS AND METHODS

Stx2 and rStxB2

Purified Stx2 was generously provided by A. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Maryland. The recombinant StxB2 (rStxB2), consisting of the StxB2 (the non-toxic binding subunit of Stx2) carrying an affinity tag of 6 consecutive histidine residues (6xHis tag) at the amino terminus, was purified with the Ni-NTA Protein Purification Kit (Qiagen, Valencia, CA). Briefly, a 290-bp fragment encoding StxB2 (minus the start codon ATG) was PCR amplified from *E. coli* O157:H7 strain 86-24 (49) by using primers *stx2B*-F (5'GGTACCAAGAAGATGTTTATGGCGGTTTTA3') and *stx2B*-R (5'GTCGACTCAGTCATTATTAAACTGCACTTCAG3'). The PCR amplified fragment was digested with *Bam*H1 and *Sal*1 and cloned at the corresponding restriction sites of the expression vector pQE-80L. The recombinant plasmid *stx2B*-pQE80L was transformed into *Escherichia coli* M15. For rStxB2 expression, *E. coli* M15 containing *stx2B*-pQE80L was inoculated into Luria-Bertani broth supplemented with ampicillin (100 μg/ml) and incubated at 37°C with shaking (250 rpm). Once the culture reached an optical density (OD_{600}) of 0.6, isopropyl-β-D-1-thiogalactopyranoside **(**IPTG; 1 mM) was added to induce transcription of *stx2B* leading to enhanced Stx2B expression. After 4 hours of growth in the presence of IPTG, the bacterial cells were collected by centrifugation (4000 x g, 20 minutes), resuspended in ice-cold phosphate buffered saline (PBS) and subjected to sonication (total of 6 pulses with each pulse lasting 20 sec). The sonicated cells were centrifuged (10,000 x g, 30 minutes, 4°C) to remove intact cells and cell debris. The supernatant was collected and applied to the Ni-NTA resin columns to bind the 6xHis tag on the rStxB2. The columns were

then washed and the protein eluted from the columns according to kit instructions. The total protein concentration of the preparation was estimated by using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The purity of the rStxB2 was assessed by SDS-PAGE and Western blot.

Isolation of Porcine Granulocytes

Blood samples were collected in heparin tubes (Becton Dickinson, Franklin Lakes, NJ) from eight healthy pigs (≥6 months old) maintained at the National Animal Disease Center, Ames, IA, during the course of this study. Whole blood smears were prepared and stained with Wright's stain (Sigma) for differential counts. Granulocytes were isolated under sterile conditions by density gradient centrifugation over Ficoll-Paque Plus (GE Life Sciences, Piscataway, NJ) as described elsewhere (Winter 2005) with minor modifications. Heparinized whole blood (15 ml) was mixed with an equal volume of 1X Hank's balanced salt solution (HBSS). After centrifugation (400 x g, 30 minutes, 18°C), the plasma, buffy coat, and Ficoll layers were removed with a serological pipet and discarded. To minimize the number of contaminating lymphocytes and monocytes in the final preparation, the lower portion of the erythrocyte/granulocyte layer was removed with a clean pipette and transferred to a clean tube. Erythrocytes were removed from the granulocyte layer by hypotonic lysis with 27 ml of sterile dH_2O followed by the addition of 3 ml 10X HBSS to restore isotonicity and centrifuged (600 x g, 20 minutes, 18°C). The lysis step was repeated, granulocytes were washed in 40 ml 1X HBSS, centrifuged (400 x g, 10 minutes, 18°C) and resuspended in either flow assay buffer (phosphate buffered saline containing 1% fetal calf serum [Hyclone, Logan, UT; heat inactivated], and 1% normal goat serum [Vector Laboratories, Burlingame,

CA]) or cell culture medium (RPMI 1640 [Invitrogen] containing 10% fetal calf serum, 3 μm beta-mercaptoethanol, penicillin and streptomycin [Sigma-Aldrich Corp., St. Louis, MO; 100 IU/ml and 100 μg/ml, respectively]). Cell concentrations were determined using a hemacytometer. Viability was assessed by trypan blue (Sigma-Aldrich) exclusion and an initial viability of ≥90% was considered the minimum acceptable value. Smears prepared from the granulocyte preparations were stained with Wright's stain (Sigma-Aldrich) for differential counts.

Flow Cytometric Evaluation of Stx2 and rStxB2 Binding and Gb_3/CD77 Expression by Porcine Granulocytes

Stx2 binding to porcine granulocytes at 4°C

In the previously-described rapid flow cytometric assay for detecting Stx binding to isolated porcine granulocytes (135), bound Stx is detected using an indirect immunofluorescence assay in which primary and secondary detection antibodies are combined in a single incubation step, rather than each reagent being added in separate incubations with additional wash steps. Although the rapid procedure reduces the handling of the granulocytes and the time needed to complete the assay, cross-reactions or reduced staining may occur when the detection reagents are combined. The rapid Stx binding assay uses constant shaking for the Stx2 incubation step. However, incubation of the granulocytes on a rotary shaker is not suitable for all of the planned studies.

To establish the 4°C Stx2 binding assay, pilot experiments were conducted to determine if Stx2 binding is affected by the additional time and handling from a sequential assay and if

shaking incubation is required for Stx2 binding. The Stx2 binding assays were modified and performed as described previously (Winter 2005). Two independent assays, each performed with granulocytes from 2 animals (4 animals total), were used to compare sequential and rapid Stx detection methods and to determine if Stx2 incubations can be performed without shaking. Isolated porcine granulocytes were diluted to a concentration of 4×10^6 cells/ml in flow assay buffer (described above). The diluted cells (50 µl) were added to 96-well plates (Corning Costar, Lowell, MA) containing 100 µl of Stx2 (750 ng/ml, final well concentration) also diluted in flow assay buffer. Flow assay buffer was used for all subsequent dilutions and washes. All incubations and washes were performed at 4°C. The granulocytes were incubated with Stx2 for 1 hour, either with constant agitation on a microplate shaker (300 rpm) or statically, and then washed twice to remove unbound Stx2. For wash steps, the plates were centrifuged (400 x g, 3 minutes), the supernatant was decanted, and the granulocytes were resuspended in buffer (200 µl). The granulocytes were then sequentially incubated with rabbit anti-Stx2 antiserum (final dilution per well was 1:1000, provided by A. O'Brien), washed, and incubated (30 minutes) with goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) secondary antibodies (Southern Biotech, Birmingham, AL, final concentration per well was 4 µg/ml). For the rapid detection assay, the granulocytes were incubated for 30 minutes with primary and secondary antibodies in a single step. The granulocytes were washed twice and resuspended in 1X FACSLyse (Becton Dickinson) to fix the cells. All samples were tested in duplicate. Negative controls and secondary antibody controls were included in each assay. Granulocytes were incubated with flow assay buffer only for the negative controls and without the primary antibody (rabbit anti-Stx2 antiserum) for the secondary antibody controls. Granulocytes were analyzed within two hours by flow

cytometry and Stx2 binding was measured by the mean fluorescence intensity (MFI) and the percentage of positive granulocytes. Static Stx2 incubations and sequential incubations with primary and secondary Stx detection reagents were used in subsequent assays and hereafter referred to as the Stx2 binding assay.

Flow cytometric analysis

A LSR II flow cytometer (Becton Dickinson) was used to acquire 10,000 events per sample. Granulocytes were initially analyzed for light scatter characteristics to determine size (forward scatter or FSC) and granularity (side scatter or SSC). Different cell populations within the isolated granulocytes were gated separately according to SSC signals. Secondary antibody and isotype controls were used to set analysis gates so that $\leq$2% of the cells in the control samples were positive. All flow cytometry data was analyzed with FlowJo software (Tree Star, Inc., Ashland OR).

Fluorescence microscopic evaluation of Stx2 binding

In some assays, Stx2 binding to isolated porcine granulocytes at 4°C was also evaluated using fluorescence microscopy. Granulocytes (1-2 µl) from two independent flow cytometric Stx2 binding assays (2 assays, 2 animals/assay) were spotted onto slides (Probe-on-Plus, Fisher Scientific, Waltham, MA) before or after the FACSlyse (Becton Dickinson) fixation step. The slides were air-dried in the dark and cover slips were mounted with mounting medium (ProLong Gold, Invitrogen, Carlsbad, CA) containing DAPI (4'-6-diamidino-2-phenylindole) to label nucleic acids. Slides were examined using an Eclipse E800

microscope (Nikon Instruments, Inc., Melville, NY). Digital images were taken with a Digital SPOT RT Slider camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using MetaVue software (Universal Imaging Corp., Downingtown, PA).

Flow cytometric rStxB2 binding assays

The binding subunit of Stx2 (Stx2B) has functional affects, which are mediated through signaling pathways initiated at the cell surface, on some cell types. Recombinant Stx2b (rStxB2) was included in this study to differentiate between effects mediated through the toxic action of the Stx2 A-subunit and those mediated by signaling pathways triggered by receptor binding.

The flow cytometric rStxB2 binding assays were performed as described for Stx2 binding. Pilot experiments with 8.7-9000 ng/ml (final well concentration) of rStxB2 and checkerboard antibody titrations were performed to determine the optimal rStxB2 concentration and saturating antibody concentrations for binding assays. The rStxB2 concentration that resulted in the highest MFI value (6000 ng/ml, final well concentration) and the antibody concentrations that resulted in the highest signal to noise ratio (rabbit anti-Stx2 antiserum at 1:100, goat anti-rabbit IgG-FITC at 4 μg/ml) were used in subsequent rStxB2 binding assays.

Binding of rStxB2 to isolated porcine granulocytes was evaluated in four independent assays with granulocytes isolated from four total animals (two animals per assay, each animal analyzed twice). The rStx2B binding assay was performed as described above for Stx2. Granulocytes were fixed with 1X FACSlyse and analyzed by flow cytometry within 2 hours.

Flow cytometric Gb_3/CD77 receptor assay

Expression of the Stx2 receptor Gb_3 ,also known as CD77, was assessed in four independent assays with granulocytes isolated from four total animals (each animal analyzed twice). The Gb_3/CD77 flow cytometry assay was performed as described (137) with some modifications. Isolated porcine granulocytes (2×10^5 cells/well) were incubated with rat anti-human CD77 clone 38.13 (20% vol/vol, AbD Serotec, Raleigh, NC), suitable for detection of Gb_3/CD77 in non-human species, and mouse anti-CD172a (10 μg/ml, VMRD Inc., Pullman, WA), included as a granulocyte marker, for 30 minutes on ice. The granulocytes were centrifuged (400 x g, 3 minutes, 4°C), washed twice in flow assay buffer (200 μl) as described above, and incubated in goat anti-rat IgM-FITC (Southern Biotech, 6 μg/ml) and goat anti-mouse IgG_1-phycoerytherin (PE) (Southern Biotech, 2 μg/ml) on ice for 30 minutes. After two additional wash steps the cells were fixed in 1X FACSLyse and analyzed by flow cytometry within two hours. Negative controls including secondary antibody and isotype controls were included in the assay.

Stx2 and rStxB2 binding to porcine granulocytes under conditions required for functional studies

For functional assays, porcine granulocytes must be incubated with Stx2 or rStxB2 in cell culture medium at 37°C. The 4°C Stx2 and rStxB2 binding assays were modified to optimize detection of Stx2 and rStxB2 binding to porcine granulocytes under these conditions. Effects of medium, temperature, and incubation time on Stx2 and rStxB2 binding were evaluated using granulocytes isolated from four animals (tested in four independent assays; two

animals per assay, 2 assays per animal. Granulocytes and Stx2 or rStxB2, diluted in flow assay buffer or cell culture medium (described above), were added to two duplicate sets of 96-well plates (4 plates/set). One set was incubated at 4°C and the second set was incubated at 37°C for 15, 30, 60, or 120 minutes. All Stx2 and rStxB2 incubations were started at the same time; washes and Stx detection steps were staggered accordingly, and performed as described above. Stx2 and rStxB2 binding were measured by the mean fluorescence intensity (MFI) and percentage of positive granulocytes.

Internalization of Stx2 and rStxB2

Four independent internalization assays were performed with granulocytes isolated from four animals (2 animals/assay, 2 assays/animal) to determine if Stx2 and rStxB2 are internalized. Porcine granulocytes were incubated with Stx2 (750 ng/ml) or rStxB2 (6000 ng/ml) in cell culture medium for 30 minutes at 4°C as described above for the binding assays. The cells were washed once, resuspended in cell culture medium only, and incubated for an additional 30 minutes at either 37°C to allow uptake of the bound proteins or at 4°C as a control. The cells were returned to or maintained at 4°C and Stx2 or rStxB2 binding was detected by flow cytometry as described.

A second type of assay was used to confirm that Stx2 and rStxB2 were internalized by porcine granulocytes. In four independent assays, granulocytes isolated from four animals (2 animals/assay, 2 assays/animal were incubated with Stx2 or rStxB2 in cell culture medium for 30 minutes at 4°C, washed once, resuspended in cell culture medium, and incubated for an additional 30 minutes at either 37°C or at 4°C as described above. Granulocytes were then

fixed with 1% paraformaldehyde (10 minutes, room temperature) and permeabilized with 1% saponin (5 minutes, room temperature) to allow detection antibodies to reach internalized Stx2 and rStxB2 as well as that on the surface. The granulocytes were incubated with detection antibodies diluted in permeabilization buffer (PBS, 1% fetal calf serum, 1% normal goat serum, 1% saponin), washed in permeabilization buffer, resuspended in 1X FACSLyse and analyzed by flow cytometry within one hour as described above.

Effect of Stx2 on Granulocyte Apoptosis

Two independent assays using granulocytes from two animals (each animal analyzed once) were performed to examine the effect of Stx2 concentration and incubation time on apoptosis. Porcine granulocytes were isolated as described above, dispensed into sterile 96-well plates, and incubated with cell culture medium alone (medium control) or with cell culture medium containing Stx2 (0.1-100 ng/ml) for 0.5, 2, 4, 6, and 18 hours of incubation at 37°C and 5% CO_2. Plates were removed from the incubator at the appropriate times, centrifuged (400 x g, 3 minutes, room temperature) and the granulocytes were resupended in 100µl annexin V binding buffer (Annexin V-FITC Apoptosis Detection Kit, Becton Dickinson). Annexin V-FITC (3 µl/well, Becton Dickinson) and propidium iodide (PI, 5µl/well, Becton Dickinson) were added to the cells and incubated for 15 minutes in the dark at room temperature. Granulocytes were analyzed within 1 hour by flow cytometry as described above. Viable cells were defined as annexin V negative and PI negative, early apoptotic cells were defined as annexin V positive and PI negative, and late apoptotic and dead cells were defined as annexin V positive and PI positive.

The effects of Stx2 and rStxB2 on porcine granulocyte apoptosis were evaluated in four independent assays using granulocytes from four animals (each animal analyzed twice). For these assays, granulocytes were incubated with Stx2 (1 ng/ml) or rStxB2 (1 ng/ml). To determine Stx-specificity of the observed effects, granulocytes were incubated with Stx2 or rStxB2 combined with anti-StxB2 (1:500, Mab BC5 BB12, provided by N. Strockbine, Centers for Disease Control and Prevention, Atlanta, Georgia) to inhibit binding. Stx2 and rStxB2 were pre-incubated with anti-StxB2 at 4°C for 2 hours before incubation with granulocytes. Granulocytes were incubated with lipopolysaccharide (LPS) from *E. coli* O111:B4 (1 ng/ml, Sigma) as a control for any LPS present in the Stx2 or rStxB2 preparations. Plates were incubated for 6 hours at 37°C and 5% CO_2, then removed from the incubator and centrifuged (400 x g, 3 minutes, room temperature). Granulocytes were incubated with apoptosis detection reagents and analyzed by flow cytometry as described above.

Effect of Stx2 on Granulocyte Oxidative Burst

The effects of Stx2 on porcine granulocyte oxidative burst were evaluated in four independent assays with granulocytes isolated from four total animals (two analyzed per assay, each animal analyzed twice). Granulocytes were incubated with medium, Stx2, rStxB2, Stx2 + Mab BC5 BB12, rStxB2 + Mab BC5 BB12, or LPS as described above for apoptosis assays. Plates were incubated for 6 hours at 37°C and 5% CO_2. The plates were removed from the incubator after six hours and were centrifuged (400 x g, 3 minutes, room temperature). Granulocytes were resuspended in 100 µl dihydrorhodamine 123 (2.5 µM, Sigma-Aldrich) and incubated at 37°C for 10 minutes. Phorbol 12-myristate 13-acetate

(PMA, 100 µl, 12.5 µg/ml, Sigma-Aldrich) was added to the cells to induce an oxidative burst followed by incubation for an additional 10 minutes at 37°C. The cells were washed once in ice-cold wash buffer, kept on ice and analyzed by flow cytometry within one hour.

Statistical Analysis

A Pearson correlation was calculated between the percentage of high SSC granulocytes determined by flow cytometry and the percentage of EOS determined by differential counts using SAS software (SAS Inc., Cary, NC). Repeated measures were collected for internalization and functional studies and data were statistically analyzed with SAS software using a General Linear Mixed Model (GLMM), a modified form of multiple regression. The independent variables of "treatment" and "day of assay" were analyzed as fixed effects and the independent variable "pig" was analyzed as a random effect. In order to determine if there were significant differences between specific treatments, t-tests were conducted as part of the GLMM. Significant results were designated as: $P \leq 0.001$ (***), $P \leq 0.01$ (**), $P \leq 0.05$ (*). Differences with $P > 0.05$ were not considered significant.

CHAPTER 4: RESULTS

Characterization of Granulocyte Preparations

Microscopic examination of Wright's stained smears of the granulocyte preparations showed that these preparations contained two cell types, EOS and PMN. Flow cytometric light scatter analysis of the granulocyte preparations differentiated two cell populations: one with a high SSC signal (2-27% of total granulocytes) and a second with a low SSC signal (73-98% of total granulocytes) (Figure 1). The percentages of high SSC and low SSC cells determined by flow cytometry correlated with the differential cell count percentages of EOS (0-27%) and PMN (73-100%), respectively, ($r = 0.61$, $P \leq 0.01$).

Stx2 Binding to Porcine Granulocytes at 4°C

Pilot studies were performed to determine if the primary and secondary antibodies used to detect bound Stx2 could be added sequentially, rather than combined as in the previously described flow cytometric Stx binding assay (137). The percentages of Stx2-positive high and low SSC granulocytes ($\geq$98% and $\geq$99%, respecitvely) and the mean fluorescence intensity (MFI) for high and low SSC granulocytes were similar with the two Stx2 detection methods (Figure 2). No differences in cell morphology or granularity profiles were detected by flow cytometric analyses. Sequential incubations with primary and secondary detection antibodies are standard practice for flow cytometry assays and were used for all subsequent assays.

The percentages and MFI of Stx2 positive granulocytes were similar after granulocytes were incubated with Stx2 for 1 hour at 4°C on a rotary shaker or statically (Figure 11, Appendix). We interpreted these results as evidence that isolated granulocytes do not need to be agitated in order to achieve Stx2 binding and static incubations were used in all subsequent assays.

Stx2 and rStxB2 Binding and Gb_3/CD77 Expression at 4°C

Flow cytometric analyses showed that both Stx2 and rStx2B bound to high SSC (≥98%) and low SSC (≥99%) granulocytes at 4°C, and that high SSC (≥94%) and low SSC (≥99%) granulocytes both expressed the Stx receptor Gb_3/CD77 (Figure 3). Fluorescence microscopic analysis of the isolated granulocytes incubated with Stx2 at 4°C clearly showed that both PMN and EOS bound Stx2 (Figure 4). The EOS were identified by their characteristic increased red autofluorescence when examined using a TRITC filter. No FITC (green) fluorescence was detected on control slides spotted with cells incubated with flow assay buffer instead of Stx2.

Stx2 Binding Under Conditions Required for Functional Studies

Similar percentages of Stx2 positive granulocytes (≥98%) were detected in samples incubated with Stx2 at 4°C or 37°C for 15 to 60 minutes, but the percentages (≥72%) of Stx2-positive granulocytes were reduced in samples incubated with Stx2 at 37°C for 120 minutes. There were no differences in the percentages of Stx2-positive granulocytes in samples incubated with Stx2 in flow assay buffer or cell culture medium.

The MFI of the high SSC granulocytes were similar for all samples incubated for 15 to 120 minutes at 4°C (Figure 5A). In contrast, the MFI of the low SSC granulocytes were similar in samples incubated with Stx2 for 15 to 60 minutes at 4°C, but increased in samples incubated 120 minutes (Figure 5C). The MFI of both the high and low SSC populations decreased over time at 37°C (Figure 5B and D). The MFI of low SSC granulocytes was similar for all granulocytes incubated up to 30 minutes at either 4°C or 37°C (Figure 5C and D). In contrast, the MFI of high SSC granulocytes was consistently higher for granulocytes incubated with Stx2 at 4°C (Figure 5A) than for those incubated at 37°C (Figure 5B).

rStxB2 Binding Under Conditions Required for Functional Studies

Overall, similar percentages of rStxB2 positive granulocytes ($\geq$97%) were detected in samples incubated with rStxB2 at 4°C or 37°C for 15 to 30 minutes, but the percentages of rStxB2-positive granulocytes ($\geq$65%) were reduced in samples incubated with rStxB2 at 37°C for 60-120 minutes. There were no differences in the percentages of rStxB2-positive granulocytes in samples incubated with rStxB2 in flow assay buffer or cell culture medium.

The MFI of the low and high SSC granulocytes increased from 15 to 120 minutes at 4°C with the exception of the high SSC granulocytes from pig 1 (Figure 6A and C). The MFI of both high and low SSC granulocytes at 37°C were reduced in comparison to high and low SSC granulocytes at 4°C (Figure 6). The MFI of the low SSC granulocytes decreased over time at 37°C (Figure 6D) while the MFI of the high SSC granulocytes were similar from 15-120 minutes at 37°C (Figure 6B).

Internalization of Stx2 and rStxB2

The reduction of Stx2 binding at 37°C described above provided presumptive evidence that Stx was internalized at 37°C. Flow cytrometry was used to compare Stx2 and rStxB2 binding on the cell surface after granulocytes were loaded with Stx2 at 4°C and then differentially incubated for an additional 30 minutes at 4°C or 37°C. Granulocytes were then incubated with detection reagents at 4°C. The MFI of granulocytes incubated at 4°C or 37°C were significantly different ($P \leq 0.001$) (Figure 7A and B).

To confirm that Stx was internalized at 37°C, similar experiments were designed to detect both internalized and surface-bound Stx2 or rStxB2. In these experiments, granulocytes were fixed and permeabilized before incubation with detection reagents. As shown in Figure 7C, the differences between the MFI of the fixed and permeabilized granulocytes incubated at 4°C or 37°C were not as pronounced as the differences observed when surface-bound Stx or rStx2B were detected (7A and B). However, these differences were still significant ($P \leq 0.01$). In this experiment, separate populations of high SSC and low SSC cells could not be detected by flow cytometry and were analyzed as one population. All of the cells had SSC cells signals comparable to those of low SSC cells, possibly due to degranulation of the high SSC cells when they were permeabilized.

Effect of Stx2 on Apoptosis in Porcine Granulocytes

Isolated porcine granulocytes were incubated for 0.5-18 hours with medium or Stx2 (0.1-100 ng/ml). When cells were incubated with medium, the percentage of early apoptotic cells in the high SSC population increased from <3% initially to >40% after 6 hours. After 18

hours, the percentage of early apoptotic high SSC granulocytes was >30% indicating that some of the early apoptotic cells had transitioned into late apoptotic or dead cells. The percentage of early apoptotic low SSC granulocytes in the medium control increased from <2% initially to >75% at 18 hours. Incubation with Stx2, at all tested concentrations, increased the percentage of early apoptotic cells compared to a medium only control after 2, 4, and 6 hours of incubation (Figure 8). The largest difference between granulocytes incubated with Stx2 or with medium was observed after 6 hours. Fewer than 2% of granulocytes were late apoptotic or dead after 0.5-6 hours of incubation with Stx2 (not shown). By 18 hours of incubation, the percentage of early apoptotic granulocytes decreased as the percentage of late apoptotic or dead granulocytes increased (not shown). Similar results were obtained for both high SSC (Fig. 8A) and low SSC (Fig. 8B) granulocytes.

Incubation of isolated granulocytes with Stx2 (1 ng/ml) for 6 hours significantly increased the mean percentage of apoptotic cells compared to a medium control for the high SSC ($P \leq 0.001$) and low SSC populations ($P \leq 0.001$) (Figure 9A and B). The effects of Stx2 on apoptosis were inhibited when Stx2 was pre-incubated with antibodies against the StxB2 to inhibit toxin binding. Incubation of granulocytes with rStxB2 (1 ng/ml), the non-toxic binding subunit, did not effect apoptosis in either cell population (Figure 9A and B). Incubation with LPS (1 ng/ml) had the opposite effect of Stx2 and significantly decreased the mean percentage of apoptotic PMN ($P \leq 0.001$) (Figure 9B).

Effect of Stx2 on the Oxidative Burst of Porcine Granulocytes

Incubation with Stx2 (1 ng/ml) for 6 hours significantly decreased the mean percentage of high SSC ($P \leq 0.001$) and low SSC ($P \leq 0.001$) granulocytes capable of an

oxidative burst induced by PMA (Figure 10A and B). The effects of Stx2 were inhibited by pre-incubation of Stx2 with antibodies against the StxB2 to inhibit toxin binding. Incubation with rStxB2 (1 ng/ml) had no effect on the oxidative burst in either high SSC or low SSC cells (Figure 10A and B). LPS (1 ng/ml) had the opposite effect of Stx2 and increased the induced oxidative burst in the high SSC ($P \leq 0.05$) and low SSC ($P \leq 0.01$) cells (Figure 10).

Figure 1. Flow cytometric side scatter (SSC) vs. forward scatter (FSC) plot of isolated porcine granulocytes showing porcine granulocyte preparations contained two cell populations distinguished by high (A) and low (B) SSC signals. Granulocytes isolated from heparinized whole blood by density gradient centrifugation were fixed and analyzed by flow cytometry.

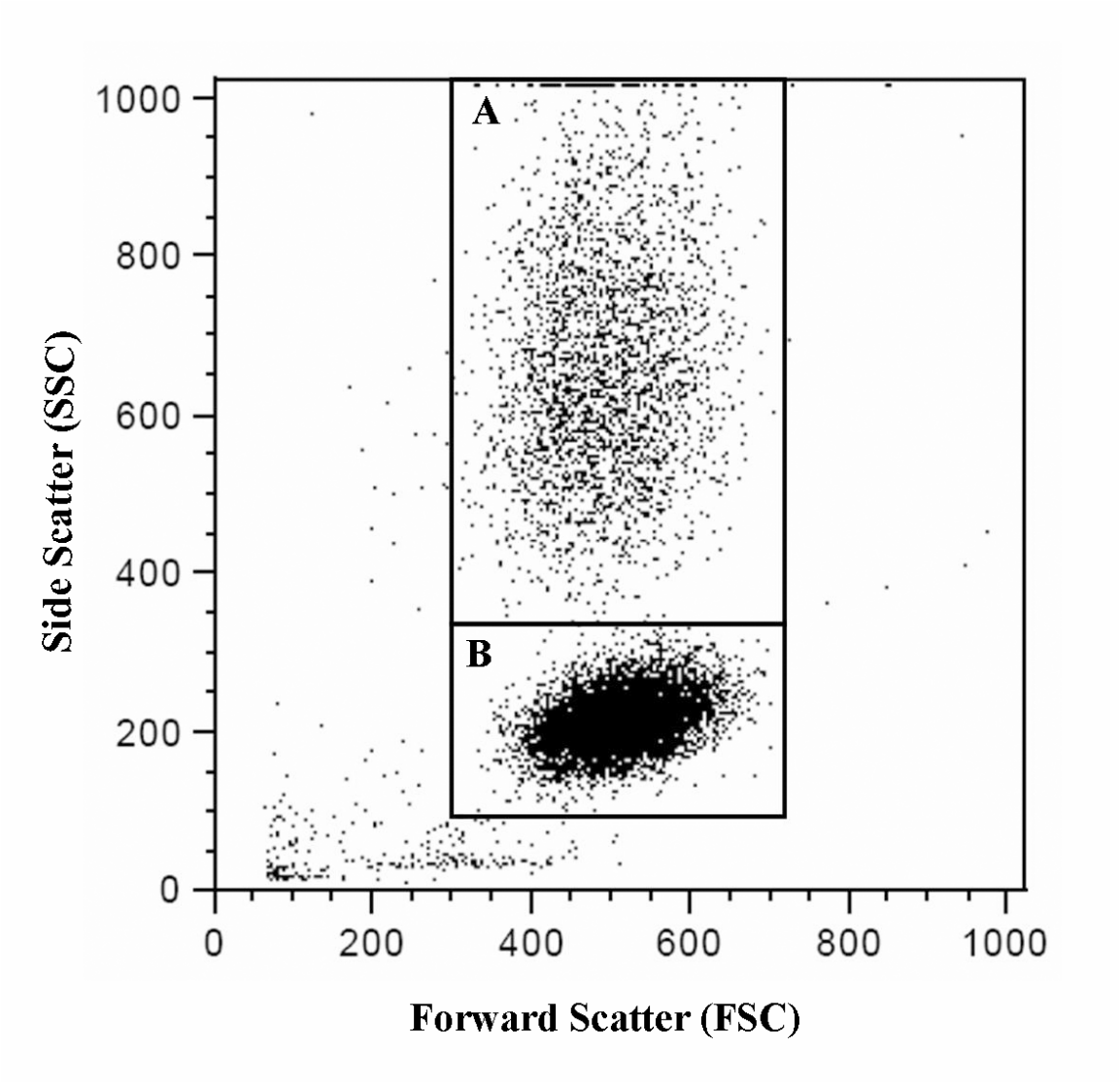
A
B
1000
800
600
400
200
0
0
200
400
600
800
1000
Side Scatter (SSC)
Forward Scatter (FSC)

Figure 2. Stx2 binding to isolated porcine granulocytes and detected by the rapid or sequential assay. Stx2 binding was detected using the rapid assay where detection reagents were combined in one incubation step (white histogram) or the sequential assay where detection reagents were incubated with the granulocytes in two separate steps (shaded histogram). Stx2 positive granulocytes with similar MFI were identified by flow cytometry with both detection methods. Stx2 bound similarly to high and low SSC granulocytes. Flow cytometry histograms are representative of results obtained for each animal (n = 4) from two independent experiments.

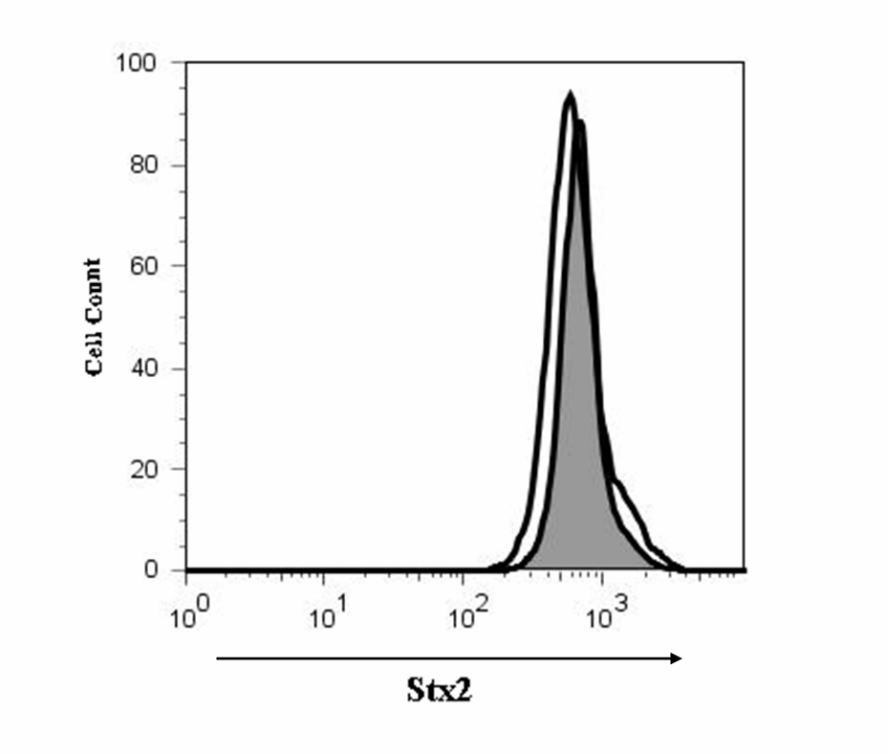
100
80
60
40
20
0
Cell Count
10^0
10^1
10^2
10^3
Stx2

Figure 3. Stx2 and rStxB2 binding and Gb_3/CD77 expression by isolated porcine granulocytes at 4°C. To detect Stx2 or rStxB2 binding, isolated granulocytes were incubated with Stx2 or rStxB2 for 1 hour, at 4°C, washed, incubated with primary and secondary detection antibodies in two separate steps, washed, fixed and analyzed by flow cytometry. To detect Gb_3/CD77 expression, isolated granulocytes were incubated with anti-CD77 primary antibody for 30 minutes at 4°C, washed, incubated with secondary detection antibody, washed, fixed and analyzed by flow cytometry. Flow cytometry histograms show Stx2, rStxB2 or Gb_3/CD77 detection (shaded histograms) and corresponding controls incubated without Stx2, rStxB2, or anti-CD77 (white histograms) for high SSC (A) and low SSC (B) granulocytes. Histograms are representative of results obtained for each animal (n = 4) from four independent experiments (each animal sampled twice and tested on separate days) .

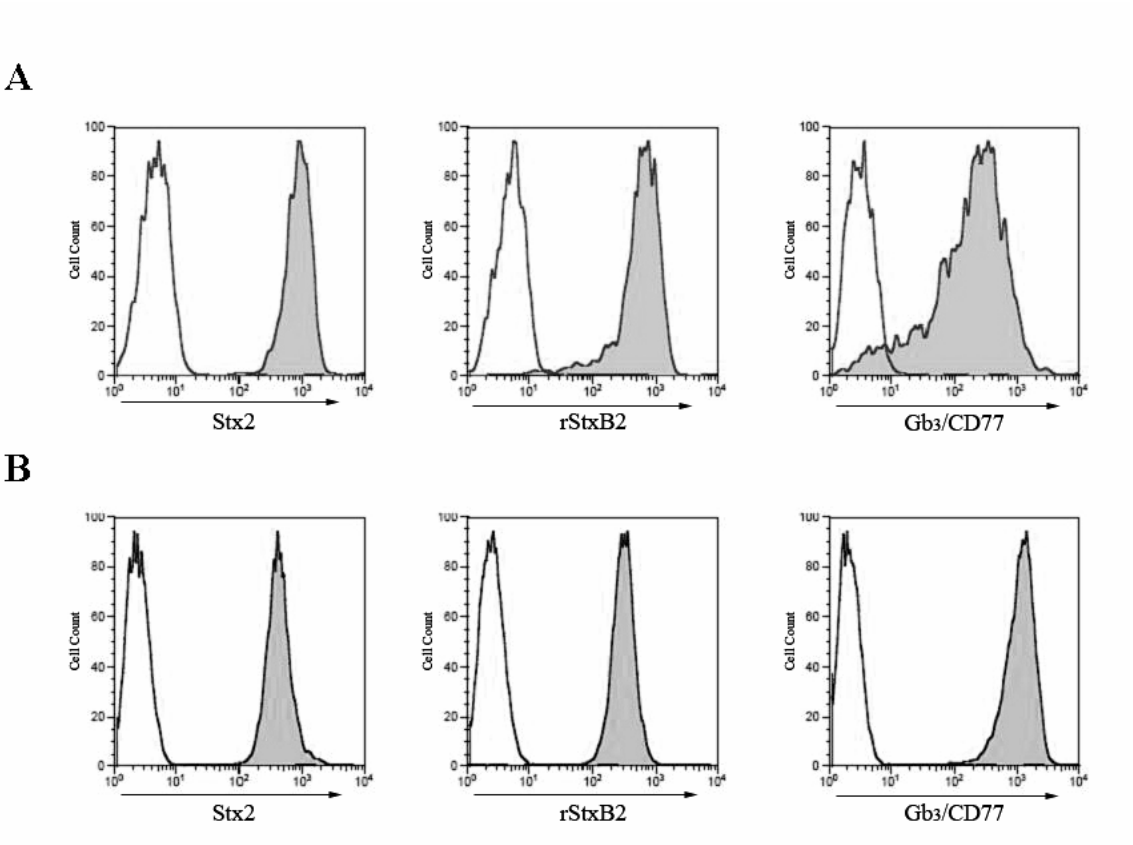
A
Cell Count
Stx2
rStxB2
Gb3/CD77
B
Cell Count
Stx2
rStxB2
Gb3/CD77

Figure 4. Fluorescence microscopic analysis of Stx2 binding to isolated porcine granulocytes. Granulocytes were incubated with Stx2 at 4°C for 30 minutes, washed, incubated with Stx2 detection reagents and spotted onto slides for evaluation. Bound Stx2 was identified by green fluorescence. EOS (indicated with arrows) were differentiated from PMN by their characteristic intense red autofluorescence when examined with a tetramethyl rhodamine isothiocyanate (TRITC) filter. DAPI was included for visualization of nucleic acid. Figure shows representative results from two independent assays with 4 animals.

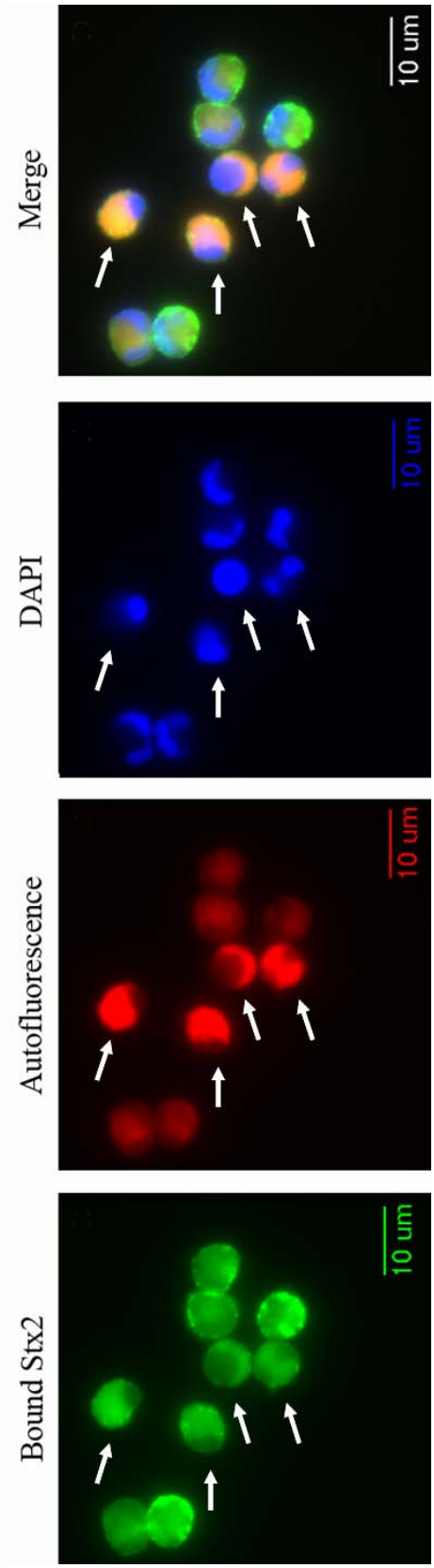
Bound Stx2
Autofluorescence
DAPI
Merge
10 um
10 um
10 um
10 um

Figure 5. Stx2 binding to high SSC (A and B) and low SSC (C and D) granulocytes at 4°C (A and C) and 37°C (B and D). Isolated porcine granulocytes were incubated with Stx2 at 4°C (A and C) or 37°C (B and D) in cell culture media for 15-120 minutes before incubation with Stx2 detection reagents, fixation, and analysis. Graphs show MFI data for each animal (n = 4) from four independent experiments (each animal sampled twice and tested on separate days)

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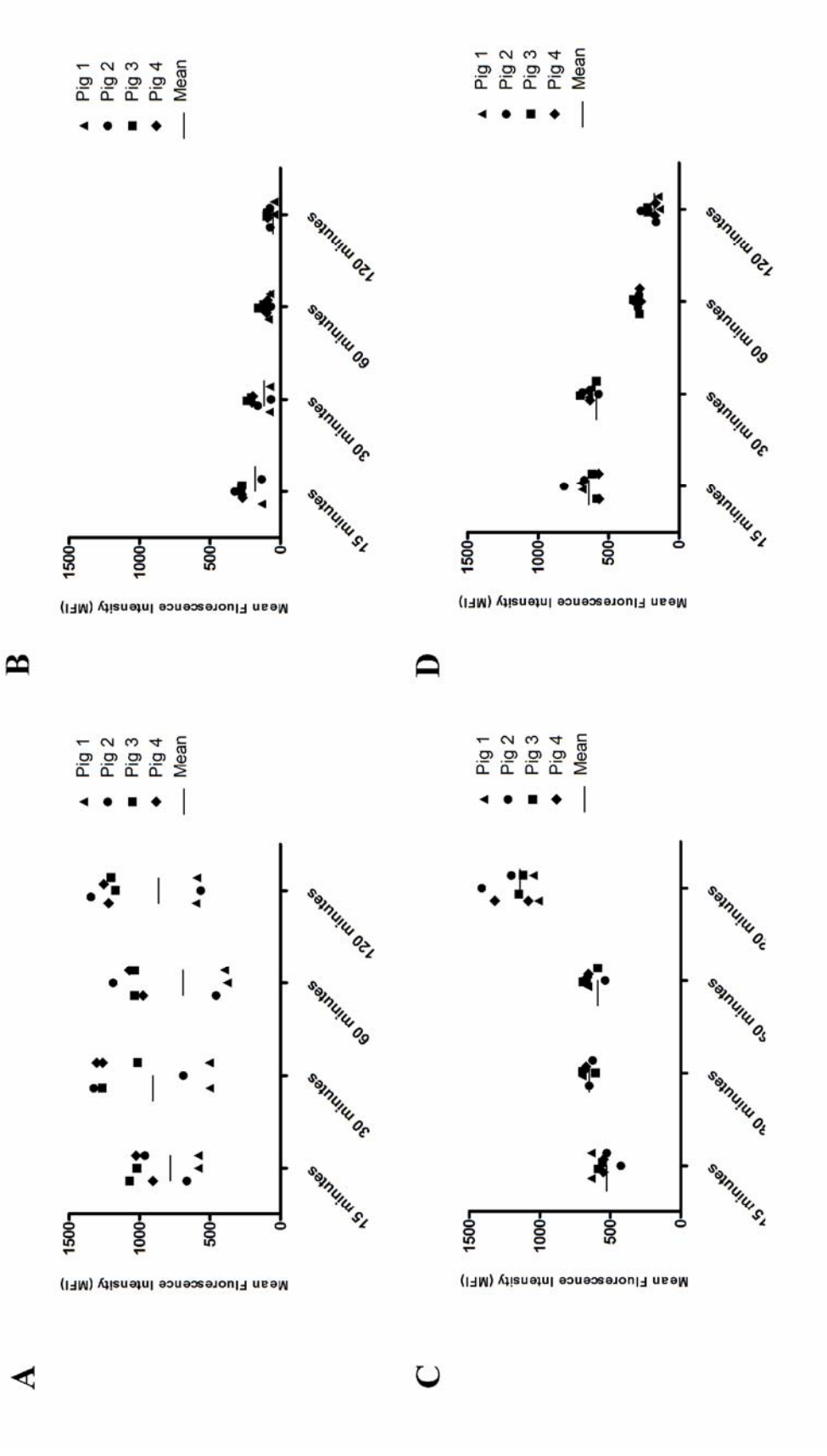

A
B
C
D
Pig 1
Pig 2
Pig 3
Pig 4
Mean
Mean Fluorescence Intensity (MFI)
1500
1000
500
0
15 minutes
30 minutes
60 minutes
120 minutes

Figure 6. rStxB2 binding to high SSC (A and B) and low SSC (C and D) granulocytes at 4°C (A and C) and 37°C (B and D). Inset graphs show the MFI data for high and low SSC granulocytes at 37°C with a decreased y-axis scale. Isolated porcine granulocytes were incubated with rStxB2 at 4°C (A and C) or 37°C (B and D) in cell culture media for 15-120 minutes before incubation with rStxB2 detection reagents, fixation, and analysis. Graphs show MFI data for each animal (n = 4) from four independent experiments (each animal sampled twice and tested on separate days).

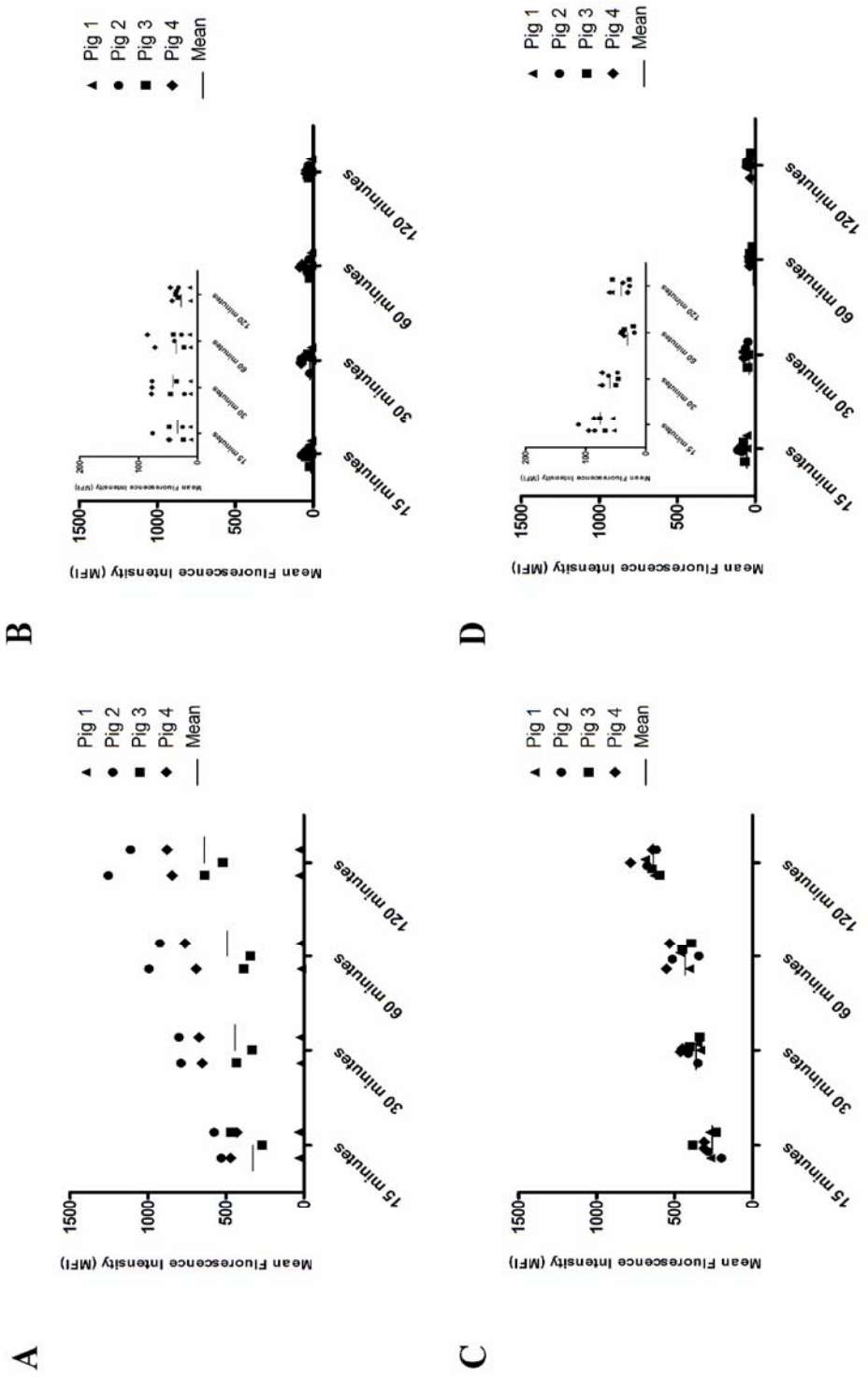

A
B
C
D
Pig 1
Pig 2
Pig 3
Pig 4
Mean
Mean Fluorescence Intensity (MFI)
1500
1000
500
0
15 minutes
30 minutes
60 minutes
120 minutes

Figure 7. Internalization of Stx2 and rStxB2 by isolated porcine granulocytes. Granulocytes were loaded with Stx2 or rStxB2 at 4°C for 30 minutes, washed, and either maintained at 4°C for an additional 30 minutes (shaded histograms) or transferred to 37°C for 30 minutes (white histograms). All granulocytes were kept at 4°C for subsequent incubations with detection reagents. To identify surface bound Stx2 or rStxB2 (A and B), granulocytes were then incubated with detection reagents, fixed and analyzed by flow cytometry. To identify both surface and internalized Stx2 or rStxB2 (C), granulocytes were fixed and permeabilized before the incubation with detection reagents. Flow cytometry histograms show Stx2 or rStxB2 on the surface of high SSC (A) and low SSC (B) populations of granulocytes. It was not possible to detect separate high and low SSC populations of permeabilized granulocytes (C) because permeabilization caused degranulation of the cells. Histograms are representative of results obtained for each animal (n = 4) from four independent experiments (each animal sampled twice and tested on separate days).

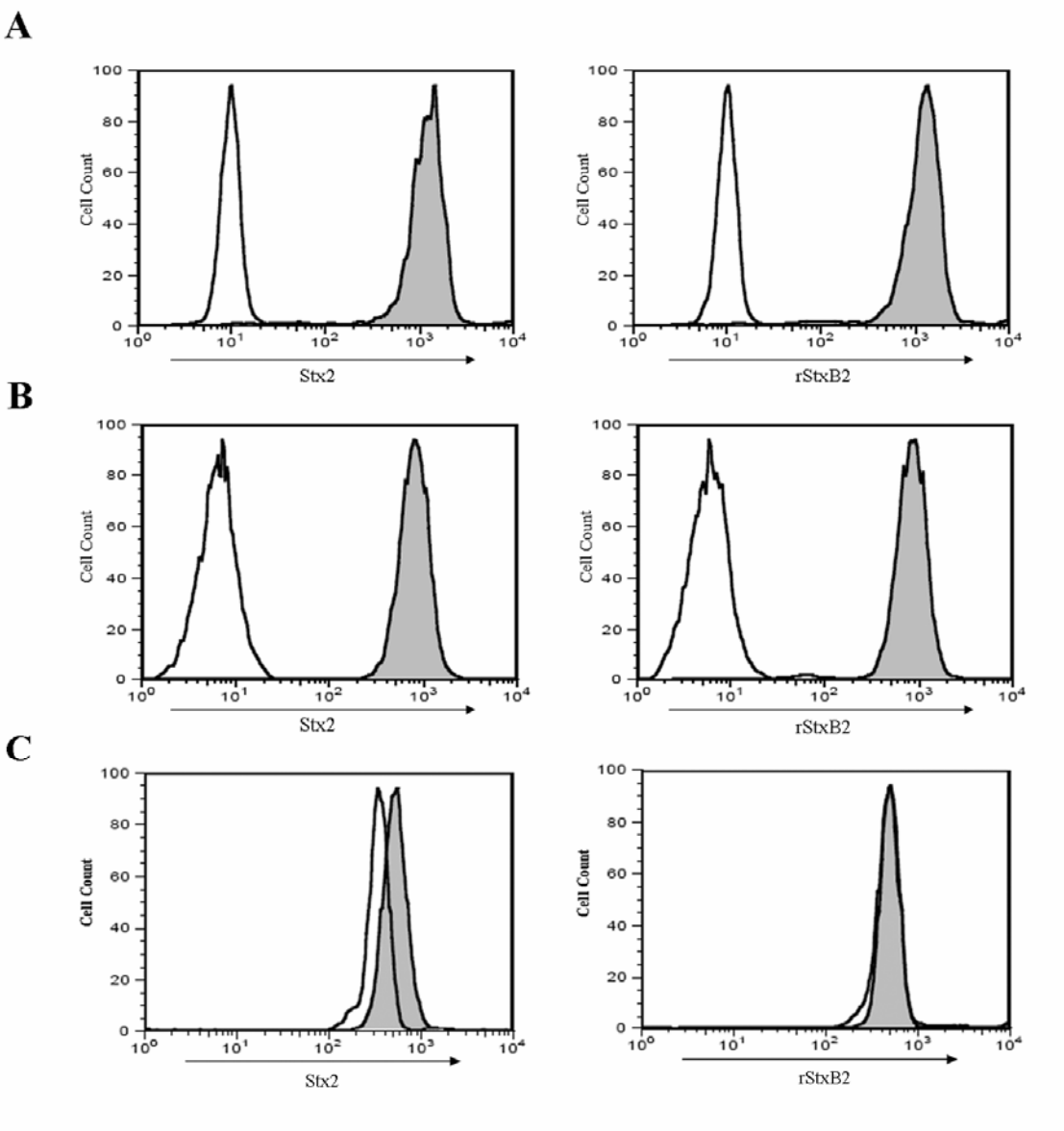

A
B
C
Cell Count
Stx2
rStxB2
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4

Figure 8. The effects of Stx2 concentration and incubation time on granulocyte apoptosis. Isolated porcine granulocytes were incubated with medium or Stx2 (0.1 – 100 ng/ml) for 0.5 – 18 hours at 37°C. The granulocytes were incubated with Annexin V-FITC and propidium iodide (PI) and early apoptotic cells were defined as Annexin V positive and PI negative. The percentage of early apoptotic cells was determined by flow cytometry analysis. The data are representative of results obtained from two independent experiments with each animal (n=4) sampled once. Results are shown for the high SSC granulocytes (A) and low SSC granulocytes (B).

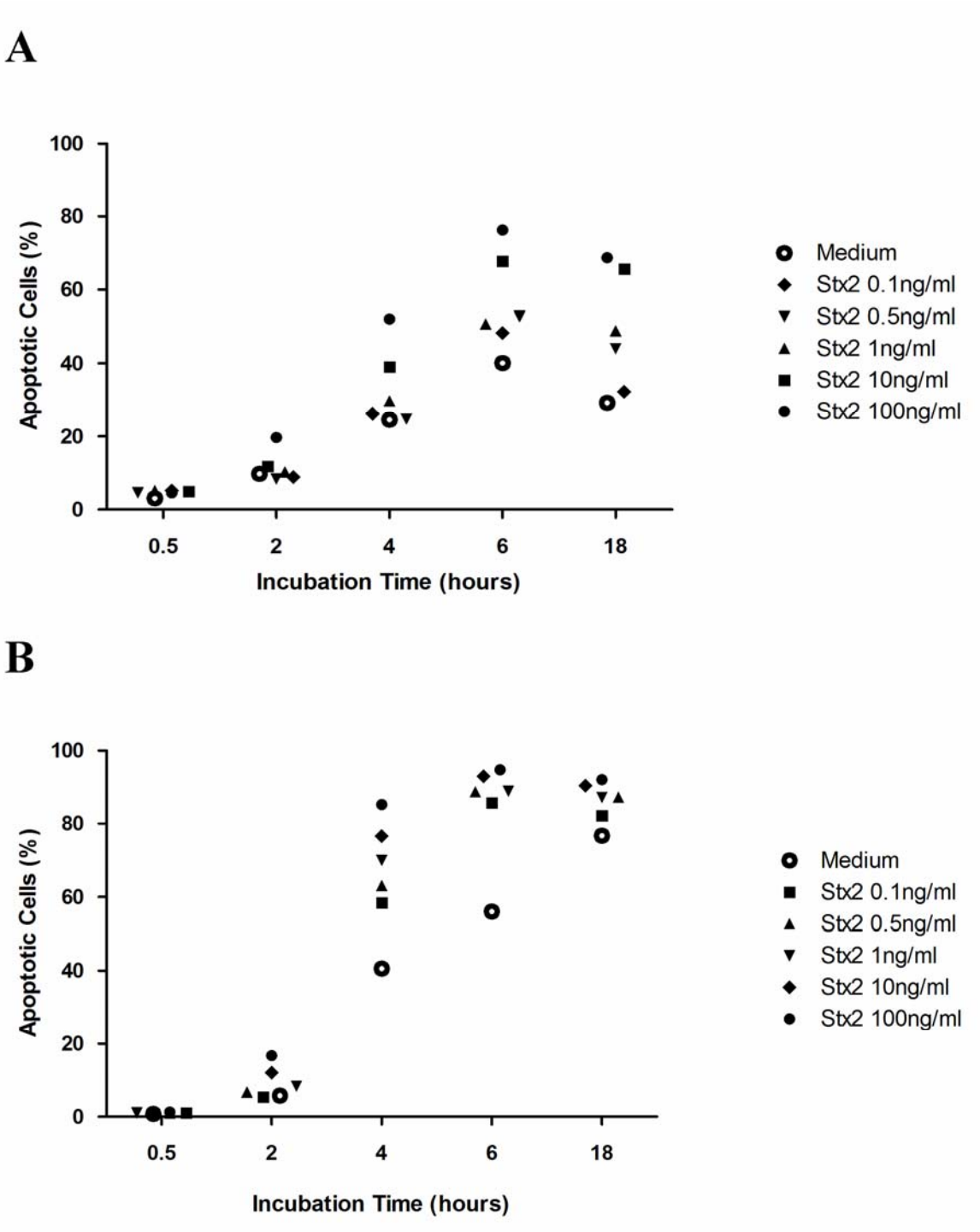

A
100
80
60
40
20
0
Apoptotic Cells (%)
0.5
2
4
6
18
Incubation Time (hours)
Medium
Stx2 0.1ng/ml
Stx2 0.5ng/ml
Stx2 1ng/ml
Stx2 10ng/ml
Stx2 100ng/ml
B
100
80
60
40
20
0
Apoptotic Cells (%)
0.5
2
4
6
18
Incubation Time (hours)
Medium
Stx2 0.1ng/ml
Stx2 0.5ng/ml
Stx2 1ng/ml
Stx2 10ng/ml
Stx2 100ng/ml

Figure 9. Effect of Stx2 on early apoptosis of isolated porcine granulocytes. Granulocytes were incubated at 37°C for 6 hours with Stx2 (1 ng/ml) or rStxB2 (1 ng/ml). To show Stx2-specific effects, some samples were incubated with Stx2 or rStxB2 pre-incubated for 2 hours with anti-StxB2 to inhibit binding. Granulocytes were incubated with medium as a negative control or LPS as a control for any LPS in the Stx2 or rStxB2 preparations. The percentage of early apoptotic cells was determined by flow cytometry as described in Fig. 8. Duplicate values for each animal (n = 4) from four independent experiments (each animal sampled twice and tested on separate days) are shown for high SSC (A) and low SSC (B) granulocytes. P values were determined using multiple regression analysis. $P \leq 0.001$ (***)

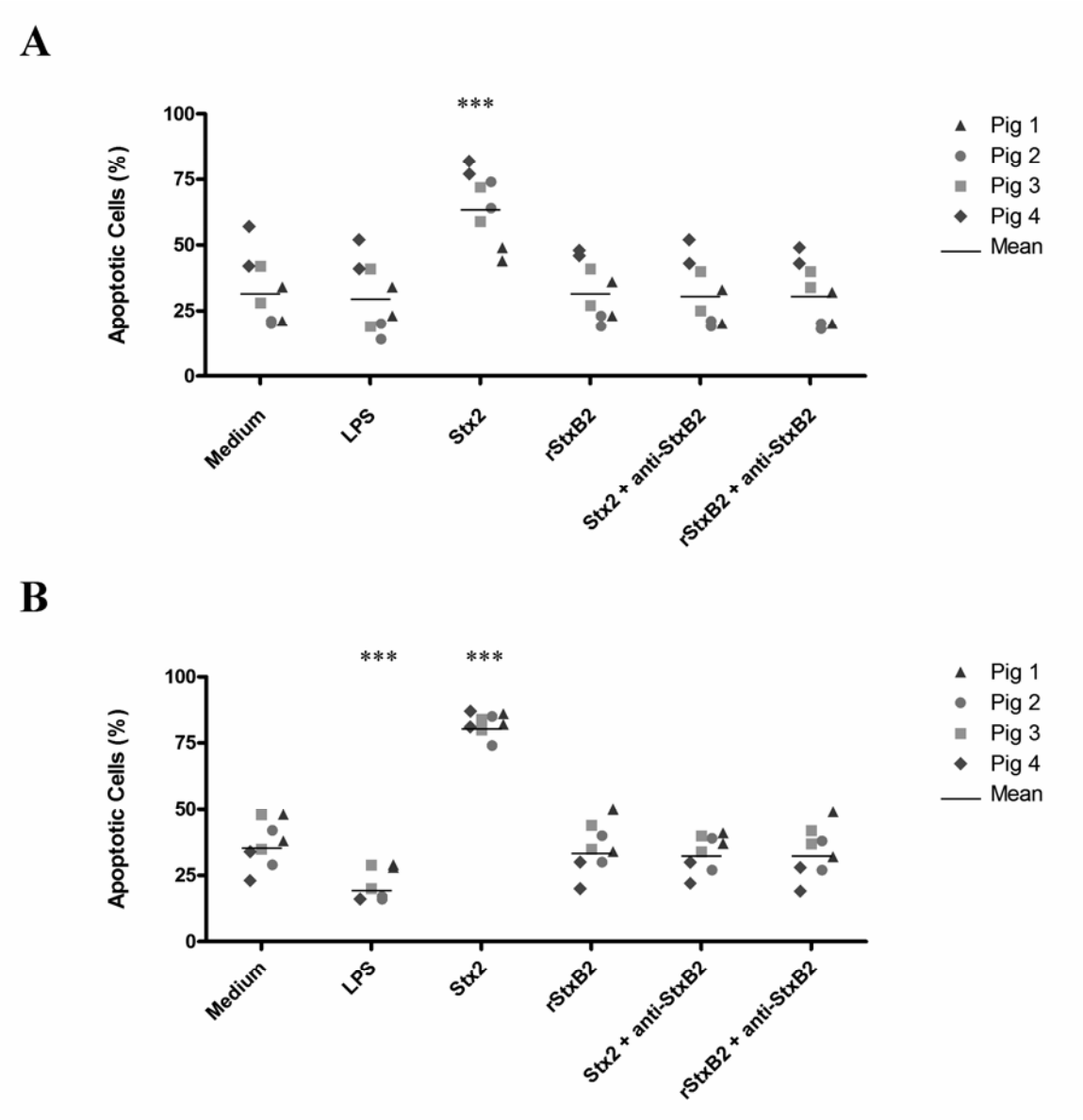
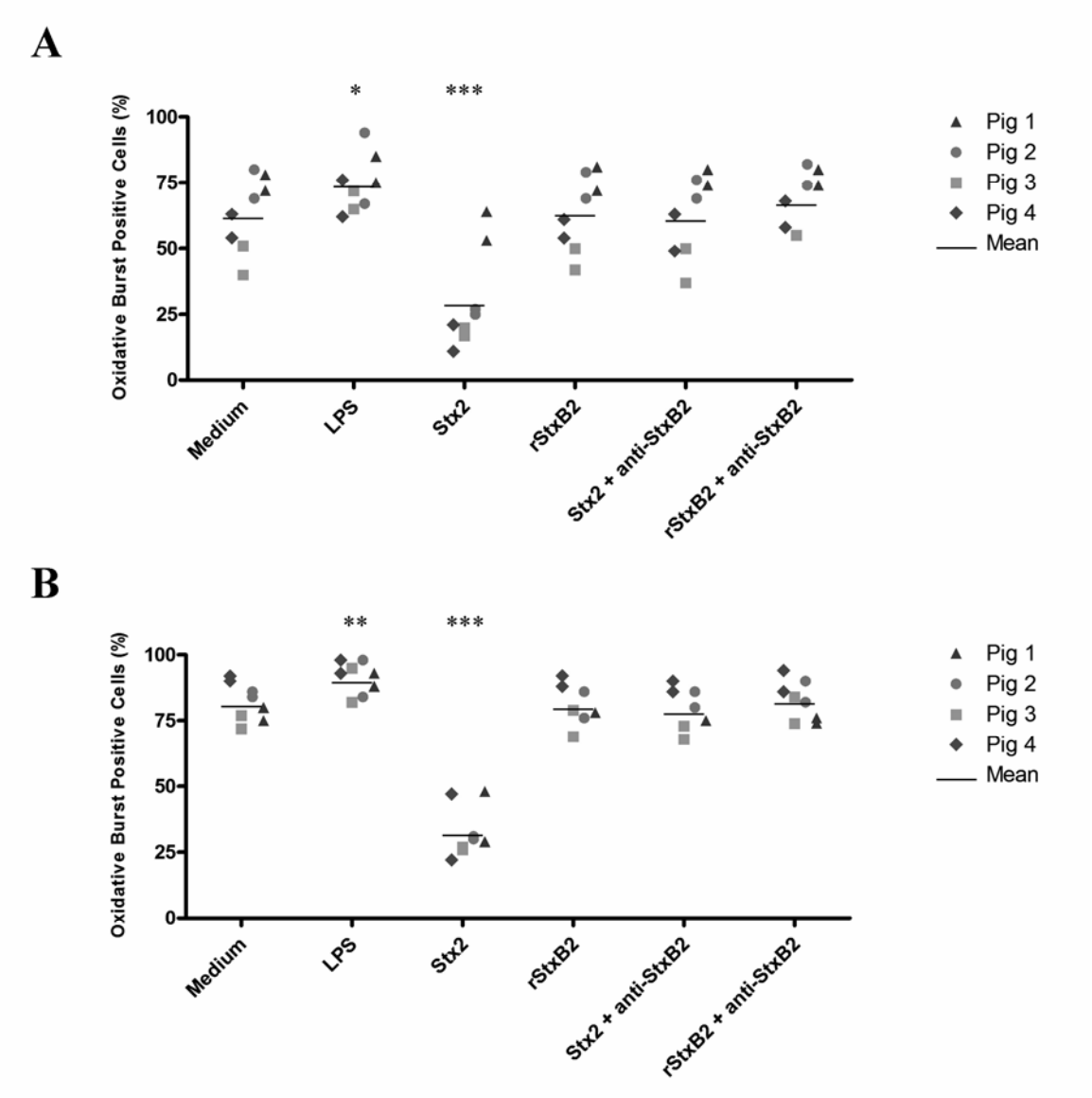

A
***
100
75
50
25
0
Apoptotic Cells (%)
Medium
LPS
Stx2
rStxB2
Stx2 + anti-StxB2
rStxB2 + anti-StxB2
Pig 1
Pig 2
Pig 3
Pig 4
Mean
B
***
***
100
75
50
25
0
Apoptotic Cells (%)
Medium
LPS
Stx2
rStxB2
Stx2 + anti-StxB2
rStxB2 + anti-StxB2
Pig 1
Pig 2
Pig 3
Pig 4
Mean

Figure 10. Effect of Stx2 on the oxidative burst of isolated porcine granulocytes. Granulocytes were incubated at 37°C for 6 hours with Stx2 (1 ng/ml) or rStxB2 (1 ng/ml). To show Stx2-specific effects, granulocytes were incubated with Stx2 or rStxB2 pre-incubated for 2 hours with anti-StxB2 to inhibit binding. Granulocytes were incubated with medium as a negative control or LPS as a control for any LPS in the Stx2 or rStxB2 preparations. The percentage of oxidative burst positive cells was determined by flow cytometry. Duplicate values for each animal (n = 4) from four independent experiments (each animal sampled twice and tested on separate days) are shown for high SSC (A) and low SSC (B) granulocytes. P values were determined by multiple regression analysis. $P \leq 0.001$ (***), $P \leq 0.01$ (**), $P \leq 0.05$ (*)


A
Oxidative Burst Positive Cells (%)
100
75
50
25
0
*
***
Medium
LPS
Stx2
rStxB2
Stx2 + anti-StxB2
rStxB2 + anti-StxB2
Pig 1
Pig 2
Pig 3
Pig 4
Mean
B
Oxidative Burst Positive Cells (%)
100
75
50
25
0
**
***
Medium
LPS
Stx2
rStxB2
Stx2 + anti-StxB2
rStxB2 + anti-StxB2
Pig 1
Pig 2
Pig 3
Pig 4
Mean

CHAPTER 5: DISCUSSION

The results of this study confirmed that Stx2 binds to isolated porcine granulocytes and provided evidence that Stx2 is internalized by and directly affects the function of porcine granulocytes *in vitro*. These results extend the evidence that PMN play a role in the pathogenesis of Stx-mediated disease. Stx2 also bound to isolated porcine EOS and altered apoptosis and oxidative burst. To the best of our knowledge, this is the first report of Stx2 binding and functional effects on EOS of any species.

Flow cytometric analysis of the porcine granulocyte preparations used in the current study revealed the presence of two cell populations that differed in granularity. The side scatter signals of the high SSC and low SSC populations were consistent with those described for EOS (126) and PMN (80), respectively. The identity of the cells could not be absolutely determined by flow cytometry because specific antibodies for identifying porcine EOS were not available at the time of this study. EOS and PMN were the only cell types noted in the analysis of Wright's stained smears of the granulocyte preparations. The percentages of high SSC and low SSC cells determined by flow cytometry correlated significantly with the percentages of EOS and PMN, respectively, determined by differential counts. Based on this data, we interpreted the high SSC population to be EOS and the low SSC population to be PMN.

The high SSC population of granulocytes, interpreted to be EOS, bound Stx2 and expressed Gb_3/CD77. Stx2 binding and Gb_3/CD77 expression by porcine EOS were not observed in previous flow cytometric studies of Stx2 binding to porcine granulocytes (135).

Scatter analysis of the granulocyte preparations in that study revealed a single cell population although it was noted that the presence of EOS in the granulocyte gate could not be ruled out because reagents to identify porcine EOS were not available (135).

Analysis of Stx2 binding by fluorescence microscopy showed that Stx2 bound to cells with high red autofluorescence when examined under TRITC filters, interpreted to be EOS, and to cells without this autofluroescense (PMN). Stx2 binding to porcine EOS was not observed in previous studies of Stx2 binding to porcine granulocytes analyzed by fluorescence microscopy (135, 136). This discrepancy may be explained by a difference in the methods used to detect Stx2 binding. While both studies used fluorescence microscopy, earlier investigations used porcine granulocyte smears that were air-dried prior to incubation with Stx2 and detection reagents (135, 136). For similar investigations in the present study, viable granulocytes were incubated with Stx2 and detection reagents before being spotted onto slides.

EOS have traditionally been regarded as cells primarily involved in allergies and defense against parasitic infections but recent studies show that EOS are involved in antigen presentation, localized inflammatory responses and regulation of the adaptive immune response (1, 55, 73, 99). Given the broad role of these cells in the immune response and that the majority of EOS naturally reside in the gastrointestinal tract (1, 55, 73), any possible involvement in Stx-mediated disease should be investigated further.

Since granulocyte functional assays require incubations at 37°C in cell culture media, one objective of this study was to ensure that Stx2 and rStxB2 bind to porcine granulocytes under these conditions as they do at 4°C (135). We demonstrated that Stx2 and rStxB2 bound to granulocytes at both temperatures and the percentage of positive cells was similar for

granulocytes incubated in either flow assay buffer or cell culture medium. We also observed a decrease in MFI when granulocytes were incubated with Stx2 or rStxB2 at 37°C. This observation led us to hypothesize that Stx2 and rStxB2 are internalized by porcine granulocytes.

Subsequent experiments designed to test the internalization hypothesis showed that porcine granulocytes loaded with Stx2 or rStxB2 at 4°C for 30 minutes and transferred to 37°C for an additional 30 minutes had a lower MFI than granulocytes maintained at 4°C throughout. Internalization was presumptively identified by a decrease in Stx2 or rStxB2 available on the cell surface and accessible to the detection antibodies. To rule out the possibility that bound Stx2 or rStxB2 were released from the receptors at 37°C, similar experiments were performed where the granulocytes were fixed and permeabilized before incubation with the detection antibodies. Permeabilization allowed the antibodies to access internalized as well as surface bound Stx2 or rStxB2. Although there were still differences in the MFI between 4°C and 37°C they were not as pronounced as in the first set of experiments. We interpreted these results as evidence that porcine granulocytes internalized a portion of the surface-bound Stx2 and rStxB2.

Stx2 binds to Gb_3/CD77 via the B-subunits while cytotoxicity is mediated via the A-subunit after internalization and retrograde transport. While many effects of Stx are attributed to the enzymatic action of the A-subunit, the B-subunits also have functional effects on some cell types. Functional effects of StxB2 are thought to be mediated through signaling pathways initiated upon binding to receptors on the cell surface rather than through any direct action of the StxB2. Binding of StxB2 to Gb_3/CD77 can trigger intracellular signaling events (115) and can induce apoptosis in Burkitt's lymphoma cells (81). Several studies found that

the binding of anti-Gb_3/CD77 antibodies to these receptors is sufficient to trigger signaling pathways and apoptosis (113, 123). We found that porcine granulocytes internalized both Stx2 and rStxB2, but only Stx2 had functional effects on the granulocytes. This finding is consistent with the interpretation that the observed functional effects resulted from a direct action of the holotoxin and not from a signaling cascade initiated upon binding to the granulocytes.

Investigations of the interactions between human PMN and Stx have produced some conflicting results. There have been two recent independent reports that Stx binds to PMN *in vitro* (10, 48) and one report that Stx did not bind to PMN *in vitro* (36). These conflicting results can possibly be attributed to differences in the methods used in the various studies. In the latter study that failed to detect Stx binding, whole blood was incubated with Stx followed by density gradient centrifugation over Ficoll-Paque to obtain the PMN before analysis to detect binding (36). Considering the reported low affinity of the Stx receptor on human PMN (119) and the sensitivity of PMN to *ex vivo* handling (137), bound Stx may have been removed from the PMN before analysis.

Most studies of the effects of Stx on apoptosis in human granulocytes have reported delayed apoptosis (11, 80) although in one study Stx had no effect on apoptosis (74). Several investigations found that incubation of human granulocytes with Stx reduces the ability of the cells to respond to subsequent stimulation with PMA resulting in a decrease in the oxidative burst response (2, 56, 72). Only one study reported that Stx increased the oxidative burst in unstimulated human granulocytes (72), a result that could not be replicated in the subsequent investigations (2, 60). There were no major differences in the methods used for

the conflicting apoptosis and oxidative burst experiments that could possibly account for such discrepancies.

Granulocytes undergo apoptosis to maintain the number of cells in circulation and to control inflammation. It has been proposed that a Stx-mediated delay in apoptosis may extend the functional lifespan of granulocytes and increase inflammation and granulocyte-mediated tissue damage. However, in contrast to our initial hypothesis and to most studies with Stx and human granulocytes (10, 77), Stx2 dramatically increased the percentage of apoptotic porcine granulocytes. The differences between the effects of Stx on human and porcine granulocytes may be explained in part by differences in receptor expression. Porcine granulocytes express Gb_3/CD77 (136), internalize Stx, and rapidly undergo apoptosis. In contrast, human granulocytes express an unknown Stx receptor, have not been shown to internalize Stx (119) and Stx delays granulocyte apoptosis. Similar to our findings in pigs, Stx also binds to granulocytes of mice and sheep via Gb_3/CD77 and accelerates granulocyte apoptosis in these species (47, 85).

The oxidative burst is the primary mechanism by which granulocytes destroy bacteria although it can also result in host tissue damage during an inflammatory response. Incubation of human granulocytes with Stx *in vitro* reduces the ability of the cells to respond to subsequent stimulation and inhibits the oxidative burst induced by PMA (2, 56, 72). In support of our initial hypothesis, incubation with Stx2 reduced the percentage of PMA-stimulated porcine granulocytes capable of an oxidative burst. Our findings are consistent with those reported for human granulocytes incubated with Stx *in vitro* and indicate that Stx2 may also reduce the ability of porcine granulocytes to respond to PMA (2, 56, 72).

In experiments aimed at determining the effect of Stx2 on porcine granulocyte apoptosis and oxidative burst, LPS was included in order to control for any LPS present in the Stx2 and rStxB2 preparations. The finding that LPS and Stx2 have opposite effects on both apoptosis and oxidative burst rules out the possibility that any LPS in the preparations was responsible for the observed functional effects. LPS has a priming effect on human PMN that results in an increased oxidative burst and delayed apoptosis. Our results indicate LPS has a similar effect on porcine granulocytes. We did not conduct experiments to determine if LPS reduced the functional effects of Stx2 and rStxB2.

The experiments in this study were conducted with granulocytes isolated from pigs $\geq$ 6 months of age. Unlike neonatal and weaned pigs, which are used as models for STEC disease (19, 20, 38, 50, 95), older pigs generally do not develop Stx-induced symptoms (7, 16). Further studies are needed to determine if granulocytes from younger pigs are similarly affected by Stx.

The new information from this study increases our understanding of the interactions of Stx and granulocytes in pigs. The studies presented here indicate that Stx2 interacts directly with porcine granulocytes. This is, to the best of our knowledge, the first report of functional effects of Stx on porcine granulocytes and the first report of Stx binding and effects on EOS in any species. The availability of porcine STEC infection models provides an opportunity to investigate the *in vivo* significance of the functional effects reported in this study.

CHAPTER 6: CONCLUSIONS

General Summary

Stx is considered the primary virulence factor responsible for the development of hemorrhagic colitis and HUS in humans. Several lines of evidence indicate that the interaction of Stx with granulocytes plays a role in the pathogenesis of Stx-mediated disease in humans. Human granulocytes bind Stx and are thought to transport it to target organs including the CNS and kidneys. Stx delays granulocyte apoptosis and induces the oxidative burst thereby extending the functional lifespan of the cells and may result in increased tissue damage. Pigs, animals that are susceptible to natural and experimental infection with STEC strains, develop CNS and kidney lesions similar to those observed in humans although the mechanism by which Stx reaches these organs is unknown. Stx binds to isolated porcine granulocytes *in vitro*. The studies described here investigated Stx2 binding to isolated porcine granulocytes and the functional effects of this binding. The results of these studies demonstrate that Stx binds to porcine granulocytes under conditions needed for functional studies and alters granulocyte apoptosis and the oxidative burst.

Objective 1

We developed a flow cytometric assay to evaluate Stx2 binding at 4°C and under conditions suitable for PMN functional studies. The flow cytometric assay developed to detect Stx2 binding to porcine granulocytes at 4°C (135) was designed as a rapid assay procedure in which primary and secondary detection reagents are combined in the same incubation step. Although this type of assay decreases the number of times the cells are

handled, combining the detection reagents may result in cross-reactions or reduced staining. The rapid assay was compared to a sequential assay procedure with separate incubation and wash steps for each reagent. Similar percentages of Stx2 positive granulocytes were detected with both assays. There was no change in granulocyte morphology when the sequential method was used and staining was not reduced when the rapid assay was used. These results indicate that either method may be used to detect Stx2 binding to isolated porcine granulocytes without adverse effects. Sequential incubations of primary and secondary detection reagents are standard practice for flow cytometry and was the procedure chosen for the Stx binding assays.

An important step before investigating the functional effects of Stx is to ensure that Stx binds to porcine granulocytes under the conditions required for function studies as they do at 4°C. We hypothesized that Stx binds to porcine granulocytes under conditions required for functional studies (37°C in cell culture medium). This set of experiments compared Stx2 binding to high and low SSC granulocytes at 4°C and 37°C from 15-120 minutes. Stx2 bound to both high and low SSC granulocytes at 4°C and 37°C. The MFI remained constant over time when the granulocytes were incubated with Stx2 at 4°C but decreased over time when incubated at 37°C.

The observed decrease in MFI when granulocytes were incubated with Stx2 at 37°C lead us to hypothesize that Stx2 is internalized by porcine granulocytes. These studies compared the MFI of granulocytes incubated with Stx2 at 4°C, washed, then differentially incubated at 4°C or 37°C before being incubated at 4°C with detection reagents. Internalization of Stx2 presumptively resulted in less surface-bound Stx2 available to the detection antibodies and translated into a decreased MFI. Both high SSC and low SSC

granulocytes internalized Stx2 and rStxB2. The finding that both Stx2 and rStxB2 are internalized will help to determine whether any functional effects are mediated by direct action of the holotoxin or by a signaling pathway initiated upon binding to receptors on the cell surface.

Objective 2

Stx delays apoptosis and decreases the oxidative burst in PMA-stimulated human PMN. There are similarities between Stx-mediated disease in humans and pigs and Stx binds to both human and porcine granulocytes. This lead to the hypotheses that Stx delays apoptosis and impairs the oxidative burst in PMA-stimulated porcine granulocytes as it does in human granulocytes. Stx2 increased the percentage of apoptotic granulocytes and decreased the percentage of oxidative burst positive granulocytes for both high and low SSC populations. Our results did not support the hypothesis that Stx2 delays porcine granulocyte apoptosis but did support the second hypothesis that Stx2 impairs the oxidative burst response in PMA-stimulated granuloyctes.

Recommendations for Future Research

The findings described in this thesis constitute only a small part of the research needed to determine the role of granulocytes in Stx-mediated disease in pigs. Numerous studies of human HUS patients have reported that Stx can be detected bound to circulating granulocytes (9, 117, 120). In this study, Stx2 binding to porcine granulocytes was detected

in vitro under several conditions including incubation at 37°C. Stx2 binding to porcine granulocytes *in vivo* could be investigated by utilizing the existing porcine infection models.

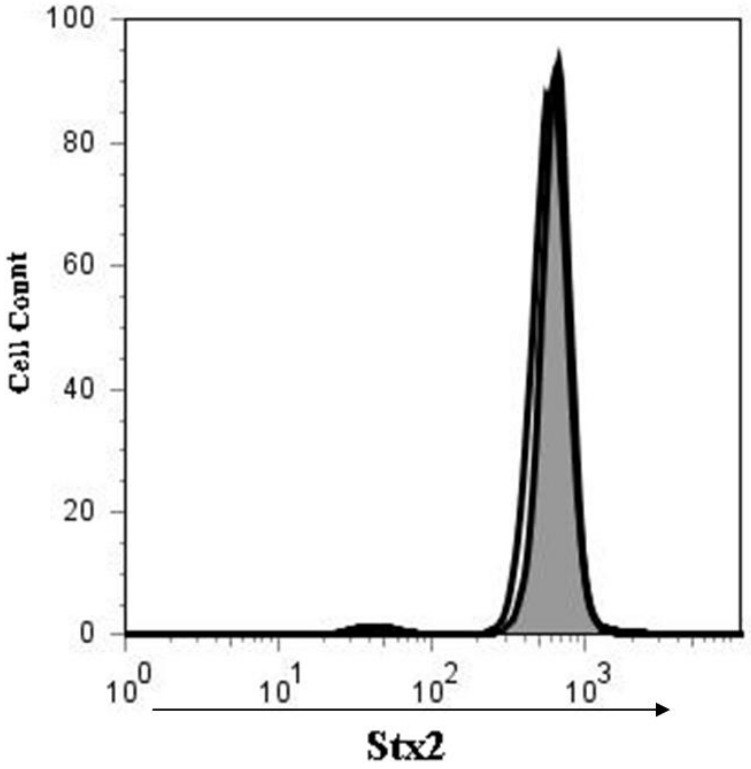
Studies should be conducted to determine if Stx bound to porcine granulocytes can be transferred to other cells expressing Gb_3/CD77. In humans, Stx is transferred from granulocytes to Stx-sensitive cells that express a high number of Gb_3/CD77 *in vitro* (119). Stx bound to granulocytes is transferred to Stx-negative granulocytes *in vitro* (10). In contrast to human granulocytes that express an unknown Stx receptor with low affinity for the toxin, porcine granulocytes express Gb_3/CD77.

We reported that Stx2 decreased the percentage of porcine granulocytes capable of an oxidative burst when stimulated with PMA. Similar results were obtained with human granulocytes when the oxidative burst was induced with PMA (2, 56, 72). However, when the oxidative burst was measured without the addition of PMA, Stx induced an oxidative burst (72). The effects of Stx2 on the oxidative burst of non-stimulated porcine granulocytes should be investigated.

The studies proposed above would provide further insight into the role of granulocytes in Stx-mediated disease in pigs and could provide insight into the mechanism by which Stx reaches target organs including the kidneys and CNS in pigs.

APPENDIX

Figure 11. Stx2 binding to isolated porcine granulocytes when the Stx2 and granulocytes were incubated together on a rotary shaker (white histogram) or statically (shaded histogram). Stx2 binding was detected using the sequential detection assay. Stx2 positive granulocytes with similar MFI were identified by flow cytometry when either incubation method was used. Stx2 bound similarly to high and low SSC granulocytes. Flow cytometry histograms are representative of results obtained for each animal (n = 4) from two independent experiments.


100
80
60
40
20
0
Cell Count
10^0
10^1
10^2
10^3
Stx2

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